MIQE & qPCR:

How to apply the MIQE Guidelines – a visual, interactive and practical qPCR guide!

Editors - Afif M. Abdel Nour & Michael W. Pfaffl

4th Edition

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Editorial

The success story of the qPCR and dPCR MIQE guidelines is continuing!

(Editorial for 4th edition published in June 2020)

by Afif M. Abdel Nour & Michael W. Pfaffl

Yes the story is continuing!

More and more researchers, biological journals, academic and commercial institutions worldwide, are supporting the MIQE guidelines. The very high recitation record of Scopus and Google Scholar documents this. As for June 2020, we count multiple thousand citations for the qPCR MIQE guideline (7.350 by Scopus and 9.800 by Google Scholar) as well as for the digital MIQE guideline (370 by Scopus and 460 by Google Scholar). Hence, the qPCR and dPCR MIQE guidelines are a worldwide standardisation success story, which are driven forward by scientific validity and credibility in the PCR community.

The present 4th edition of the MIQE & qPCR iBook will push the 'MIQE idea' even further in any laboratory worldwide and beyond in the scientists' workflow and minds. It should clearly show how to apply the guidelines and serve as a handy, visual, interactive and practical guide. Since the 2nd edition in July 2016, we provided an additional MIQE & qPCR eBook version, which is readable on any eBook reader device (ePub file). This leaded to more than 4.800 extra downloads of the MIQE & qPCR iBook and eBook from over 40 countries worldwide.

Our goal for the 4th edition is to update the existing content by new hot topics, and to create an overall fancy interactive tool, by interfacing scientific publications with educating pictures, videos and scientific talks. The editors implemented the following new chapters:

- "**ddPCR – droplet digital PCR**" authored by Afif Abdel Nour
- "**Reproducible and Sensitive Assays using 3- and 6-colour Crystal Digital PCR™ for Detecting Point Mutations in Human Breast Cancer**" authored by scientists from Stilla Technologies
- "T**he influence of plastic consumables on qPCR**" authored by application specialists by Eppendorf

In summary, we are proud to present a selection of international highly recognized authors from the academic field and from industrial research, presenting their latest research applications. The described qPCR and dPCR methods and applications are tightly linked to the MIQE context, and show it clearly based on educational questionnaires or interactive ‘how to do’ instruction sheets. The at-hand MIQE & qPCR iBook & eBook should deliver the MIQE guidelines directly to the researcher and help to solve the daily problems in the molecular biology laboratory using quantitative PCR, digital PCR, single-cell qPCR, microRNA applications or any comparable techniques using PCR.

The editors hope you like our explanatory, interactive and educational iBook, eBook and ePub concept, showing the advantages of the MIQE guidelines in an easy and understandable way. The proper application and recommendation mentioned in that publication should guarantee the successful qPCR or dPCR application in the lab, and will help authors, reviewers, editors, and researchers to evaluate the quality of the presented publication.

The editors

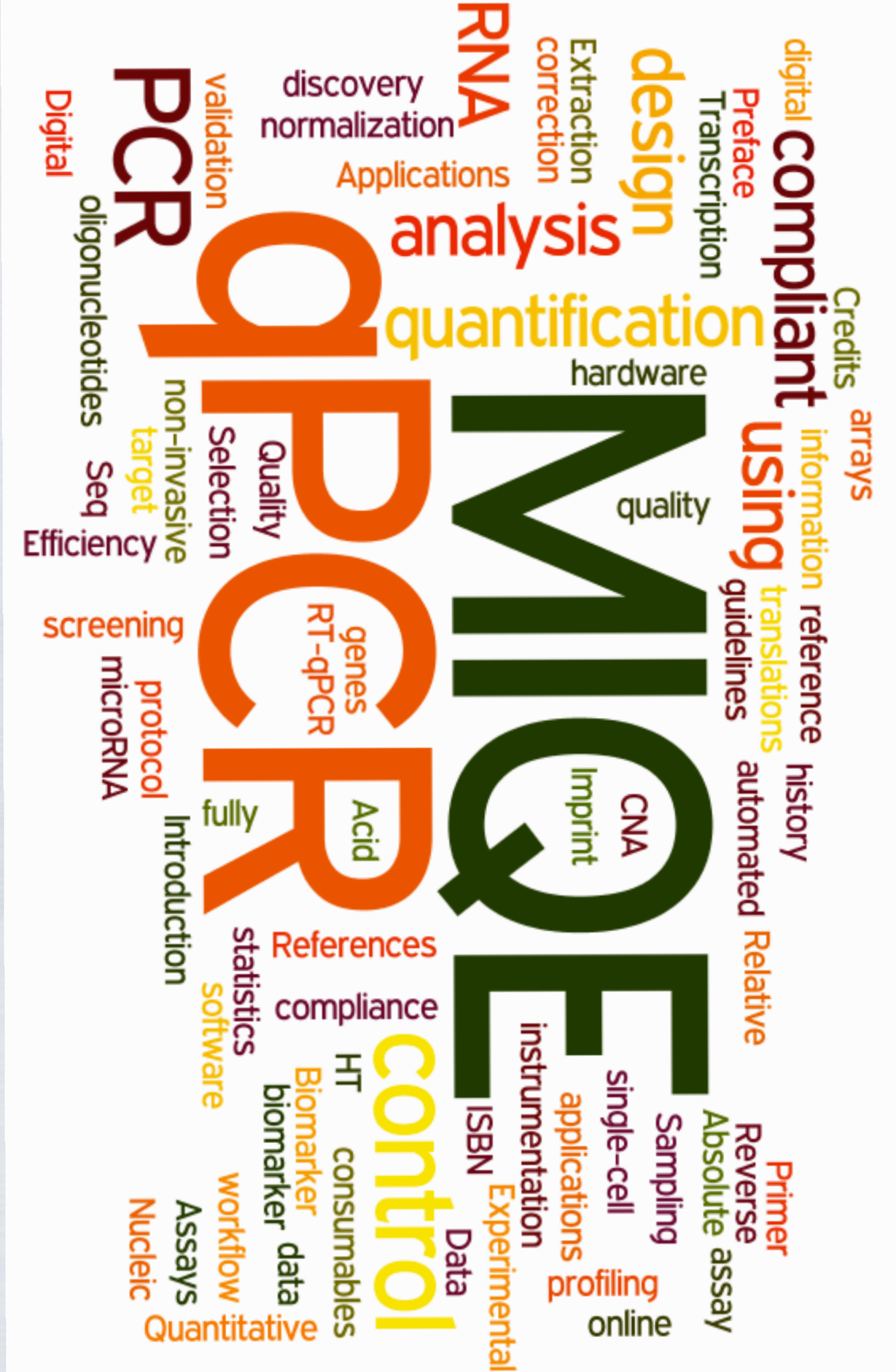
Afif M. Abdel Nour & Michael W. Pfaffl

Preface

The MIQE Guideline -- The winner is scientist's credibility

(Updated from previous editions)

Afif M. Abdel Nour & Michael W. Pfaffl



Throughout the past 37 years, the Polymerase Chain Reaction (PCR) has proven to be the most powerful technique in a scientist toolbox. Only few molecular techniques had such a success story like PCR. Maybe the best proof of its success is the implementation of PCR technology in every molecular biology laboratory routine all over the five continents. Add to that the astonishing evolution from the first generation PCR, the classical block PCR, to the 'gold standard' second generation fully quantitative "real-time quantitative PCR" (qPCR), and recently to the third and breath-taking generation high exact the "digital PCR" (dPCR).

The maturity of this technique was completed by the publication of the MIQE guideline for qPCR (1) and dPCR (2). The main objectives of those guidelines were not only to point out the pitfalls in experiments but also helping researchers to perform valid quantitative PCR experiments, and publish high-end quality papers. To help with compliance, an MIQE app has been developed for mobile devices (3), tablets and personal computers, working on various operating systems, like iOS, Android, and html5. In a study by Dooms et al. (2013), it was shown that respecting the MIQE guideline in large clinical "and pre-clinical trials would have a small but non-significant increase in the cost of performing qPCR experiments (4).

The quality of articles reporting the use of qPCR was assessed and published recently in Nature Methods (5) and PLOS One (6). In both analyses, papers were scrutinised and classified for their level of compliance to the MIQE guideline. The results were poor and pointing out the need for more rigorous implications from the editors and reviewers that must be more direct. Noteworthy, some journals have started to play the game and impose the MIQE guideline. Newly, biotech companies started to point out qPCR and dPCR products that are compliant to the MIQE, to cite some Bio-Rad Laboratories, Qiagen, Roche Life Sciences, Thermo Scientific, Sigma-Aldrich, PrimerDesign, … and more.

qPCR -- a global technique

The future aim should be to maximize the fraction of researchers applying MIQE, by working according to the guidelines and cite these in the scientific literature. In a current analysis is should shed the light on 'the good' and 'the bad' fraction of researchers, one working according the MIQE guidelines, and others not. Briefly, the Scopus database (Elsevier) was analysed in a first step for the term 'real-time PCR' over 12 years, in all papers that were published between January 2009 and May 2020 by author's affiliation and classified by country. As result, 'real-time PCR' mentioning papers were published by researcher from 219 countries and territories (figure 1). It is a surprise that those qPCR papers published were distributed unevenly, whereby China is 'publisher number one' with 21.6% of the total,

USA scored 16.9%,“and the top five western European countries (Germany, United Kingdom, France, Italy, and Spain) counted a merged interesting score of 18.6%. The remaining rest of the world shared a considerable percentage of those publications (39.5%).

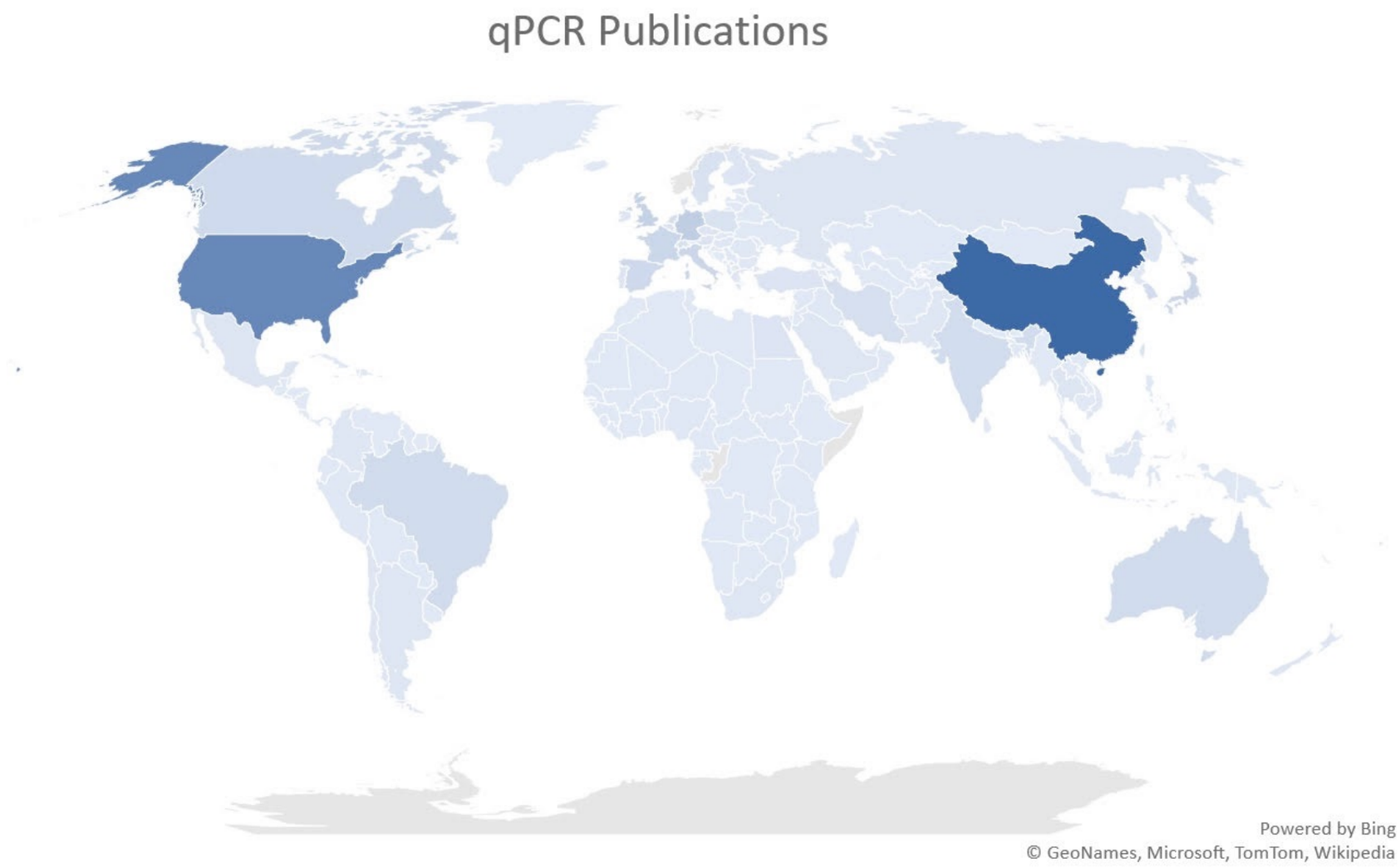
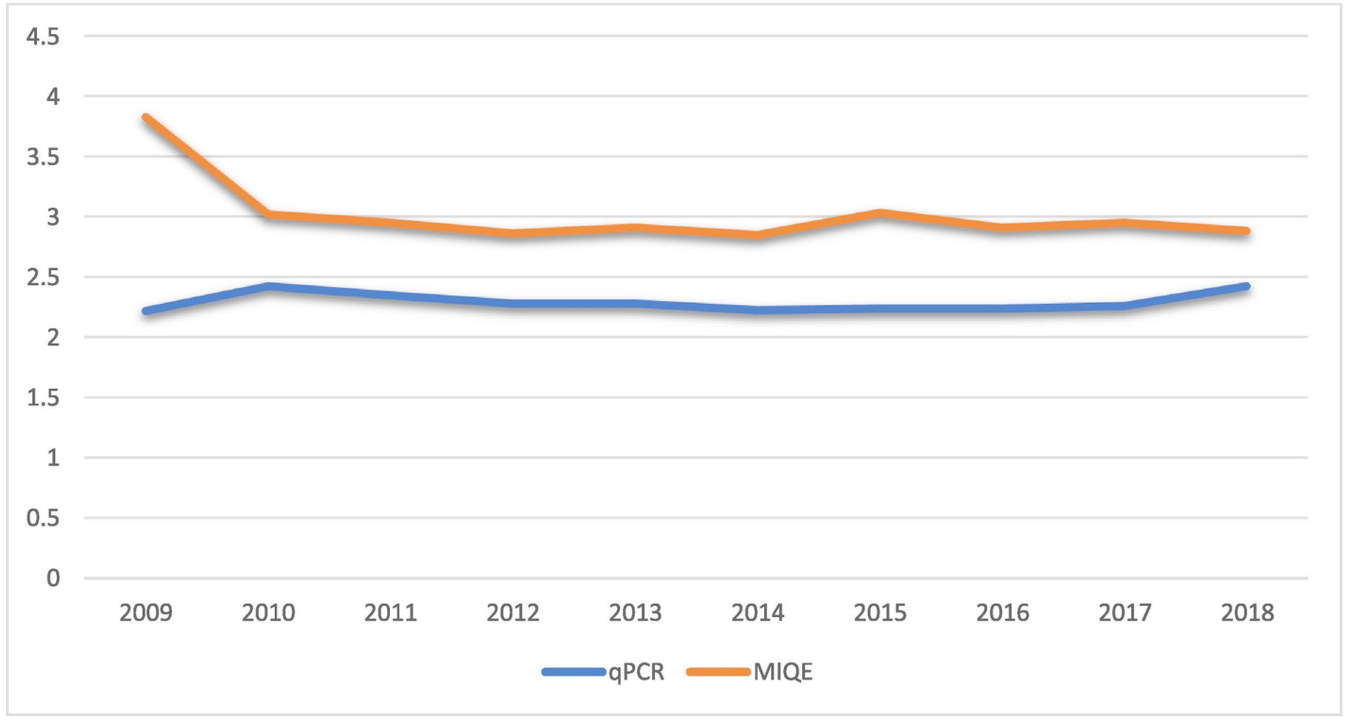


Figure 1: Worldwide distribution of scientific papers mentioning ‘real-time PCR’ (10 years overview, published from January 2009 till April 2020).

Are the MIQE-guidelines allowing researchers to publish in high CiteScore journals?

The rationale behind having guidelines is to help the scientific community to publish more reliable and reproducible data. In our hunt for an answer for the usefulness of these guidelines in publishing high-quality findings, we compared the mean weighted CiteScore for papers citing “qPCR” with those citing the “MIQE-guidelines” between 2009 and 2018. Figure below (*Mean weighted Citecore for «qPCR» and «MIQE» terms between 2009 and 2018*) shows that throughout the last 10 years, the “MIQE” papers have a higher CiteScore. Thus, researchers following the MIQE guidelines have better chances of publishing highly cited papers.



In a further step the Scopus database was screened additionally for the term of ‘MIQE’. The ratio of the ‘MIQE’ citation over ‘real-time PCR’ per country was than calculated, and should deliver a rough estimate for doing ‘good and reliable qPCR’. A fully new distribution pattern was delivered for the researchers and laboratories applying the MIQE guidelines in the scientific literature. MIQE compliance was headed by US and German publications, followed by China, UK, France, Italy and other countries, giving us hints about the overall valid qPCR practise in a worldwide comparison.

Our final aim is to democratize qPCR and the quality mark ‘MIQE guidelines’, by using this in-hand MIQE & qPCR publication, applying the authors recommendations. A further objective for the 3rd edition is to make this powerful knowledge and technology available for every laboratory worldwide interested in molecular diagnostics. It is foreseen that the increase of technical, molecular, or data analysis solutions in this broad field of qPCR offered by the biotech vendors will enhance the worldwide success. Further the drop of the costs of reagents or hardware will boost the growth of any qPCR or dPCR technology all over the world, even in the emerging markets.

The expansion of the MIQE-guidelines over time

Concomitant with our analysis of “PCR” productivity by country over the last 10 years, we screened the literature for the “MIQE” guidelines which were first introduced in 2009 to enable among others the reproducibility of published experiments. With more than 5977 citations, the MIQE-guidelines are proven to be essential for the qPCR community. However, despite the fact the qPCR users around the world are able to identify and consult the MIQE-guidelines and are convinced of their necessity, few are following them. When it comes to citations of MIQE per country, the distribution is unbalanced. Table 1 shows the top ten countries from where researchers cited the MIQE-guidelines; those countries accounted for 2/3 of the published papers. The USA is first with 13.6%, China is second with 10.8% and the big five Europeans countries totaled an outstanding percentage of 26.1%. The reasons behind this uneven balanced are: i) most of the qPCR experts that published the MIQE guidelines are from Europe, ii) those experts promoted and still are promoting those guidelines in their respective countries and universities/institutions, iii) the unprecedented support from the biotech companies some of which were promoting it either with their MIQE-compliant products or by thousands of training workshops. In contrast, the promotion for the guidelines is still at the embryonic phase in the emerging market. With the exception of a few researchers, the knowledge of those guidelines is still humble in those countries with only 2.7% of the total papers citing the term MIQE.

Table 1. Percentage of papers citing “MIQE” between 2009 and 2018 per affiliated country.

Country	MIQE citing papers
The United States of America	13.6%
China	10.8%
Germany	7.0%
The United Kingdom	5.8%
Brazil	5.2%
Italy	4.6%
Spain	4.5%
France	4.1%
Canada	3.6%
Australia	3.2%

In our quest to seek the best country that is following the MIQE-guidelines, we calculated the ratio of papers citing the “MIQE” vs “Real-Time PCR”. Overall, the worldwide ratio was very low (2.4%), and this result is reflective of the time constraint imposed by the fact that the term “MIQE” was introduced in 2009, whereas the term “qPCR” is there since 1994.

Surprisingly, Georgia has the highest ratio with 100.0%. Obviously, this result is somehow misleading if we take into consideration the total number of published papers on PCR from this country, which turned out to be only one. When considering the top countries, the big five European countries have a cumulative acceptable ratio of 4.0% followed by the USA (2.3%) and China (0.9%).

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F., Herring, E., Hoenicka, H., Icardi, L., Jaggi, R., Javad, F., Karampelias, M., Kibenge, F., Kibenge, M., Kumps, C., Lambertz, I., Lammens, T., Markey, A., Messiaen, P., Mets, E., Morais, S., Mudarra-Rubio, A., Nakiwala, J., Nelis, H., Olsvik, P. A., Perez-Novo, C., Plusquin, M., Remans, T., Rihani, A., Rodrigues-Santos, P., Rondou, P., Sanders, R., Schmidt-Bleek, K., Skovgaard, K., Smeets, K., Tabera, L., Toegel, S., Van Acker, T., Van den Broeck, W., Van der Meulen, J., Van Gele, M., Van Peer, G., Van Poucke, M., Van Roy, N., Vergult, S., Wauman, J., Tshuikina-Wiklander, M., Willems, E., Zaccara, S., Zeka, F. & Vandesompele, J. (2013). The need for transparency and good practices in the qPCR literature. Nat Methods 10, 1063-1067.

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Chapter 1

A History of the MIQE guidelines

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Section 1

Introduction

An abundance of methodologies, methods and protocols underpin the results and conclusions reported in thousands of scientific peer-reviewed publications that appear in hundreds of journals every year. They are disseminated to other researchers, often generating a vast amount of publicity once aired by television and the popular press, especially when they claim breakthroughs in medical matters. This stimulates further research, invites the investment of huge sums of money and raises hope for millions of individuals. It is not unreasonable to expect these methods to be reliable, rigorous and robust if they are to be fit for purpose. They must also be reported in a transparent manner to permit independent verification, which is the foundation of the scientific method and essential for supporting and enhancing an effective research infrastructure.

Amongst the many molecular technologies used, the polymerase chain reaction (PCR) is arguably the most important one ever invented and has been instrumental in driving developments in biology, medicine, agriculture, forensics and biotechnology. Constant development from its original incarnation as “legacy” PCR through fluorescence-based quantitative real-time PCR (qPCR) to digital PCR (dPCR) has opened up the technology’s scope, convenience and reach to even the smallest laboratory and is helping to add new applications all the time. Invariably, this popularity has a downside: the original, simple procedure has led to numerous changes that have resulted in protocols that often generate conflicting data and lead to incompatible conclusions. This invariably leads to discordance amongst

the published literature and it can often be unclear which of several published interpretations to trust.

Differences can be generic or specific:

1. Generic differences are inherent to qPCR technology, e.g. use of different instruments, choice of enzymes accompanied by manufacturer-specific reaction buffers or data analysis software based on different statistical methodology. The investigator can choose these components, but does so without exhaustive knowledge of what the detailed performance criteria of the instruments are, what the various reaction buffers contain or whether the algorithms used to report the results of an experiment are valid. Hence discordant results cannot easily be rationalised or reconciled, but have to be ascribed to different experimental set-up and tools. This makes generic differences particularly difficult to manage.

2. Specific differences are caused by the adjustments to experimental protocols, e.g. when extraction and analysis procedures developed for fresh tissue biopsies are modified for use with formalin fixed paraffin-embedded archival material. Similarly, investigators can achieve conflicting results based on the use of different cDNA priming methods or by targeting splice variants of an mRNA. Such adaptations inevitably lead to differences, but they are directly under the control of the investigator and variable results can be rationalised by careful attention to experimental detail.

An important consequence of these differences is that any reporting of results and conclusions should be accompanied by detailed and complete technical information, allowing the reader of a publication to either reproduce results or reconcile any discordance.

Section 2

Identification of the problem

In 1998, we published a review entitled " Molecular assessment of tumour stage and disease recurrence using PCR-based assays", in which we discussed the use of PCR-based assays as potential aids in the clinical management of cancer patients (Bustin and Dorudi, 1998). We pointed out that "like every new technology, PCR has built-in technical and biological limitations that must be recognised and addressed if it is to become a routine tool in clinical diagnostics and that "the use of multiple protocols prevents any standardisation and introduces numerous potential sources of technical error". With regards to the reverse transcription (RT)-PCR, we commented that "the efficiency of the RT step itself is extremely variable and differences in tissue-processing methods and PCR protocols have been shown to influence its sensitivity and specificity directly". Our conclusion was that "the wave of recent enthusiasm for these methodologies needs to be tempered until optimal standardised protocols for each of the common cancers are identified".

Another review published a few months later discussed in detail the pitfalls and potential associated with quantitative RT-PCR (Freeman et al., 1999). It concluded that its use required a comprehensive knowledge of its technical aspects as well as correction for experimental variations in individual RT and PCR efficiencies and described this area of research as " frequently confusing and contradictory".

This period witnessed the first large-scale deployment of qPCR instruments, which were being used to transform the practice of PCR. It is difficult to convey to

today's young researchers the tremendous sense of excitement that accompanied the introduction of these tools. They made PCR a high throughput, quantitative technology that allowed the rapid accumulation of large amounts of data without requiring much hands-on time, tedious pouring and running of gels, followed by photography and analysis of bands that might or might not be present and often were visible to the naked eye but somehow managed to disappear from the photograph. Photography was on instant Polaroid film in the more prosperous labs, but could also involve the manual spooling of film onto reels and its development and printing in one's own dark room. Hence setting up a 96 well plate qPCR run on an ABI 7700 and coming back a couple of hours later to see amplification plots and quantification cycles was nothing short of miraculous.

The first review to focus on the complexity of qPCR, and RT-qPCR in particular, was published in 2000 and has become the most cited review in this field (Bustin, 2000). It discussed the substantial problems associated with the technique's true sensitivity, reproducibility and specificity as well as the problems inherent in the quantitative aspect of this new PCR technology. It suggested that qPCR promised to overcome many of the limitations of legacy PCR, but that its successful application depended on a clear understanding of the practical problems inherent to this method, and careful experimental design, application and validation.

The following pages present an overview of the main areas that require the attention of anyone using qPCR, chart the increasing awareness of the specific problems associated with the different categories, identify how the egregious application of qPCR in the mumps, measles and rubella (MMR)/autism debate stimulated the publication of the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines and end with an assessment of the impact they have had.

Section 3

Sample information

Every qPCR experiment starts with the identification, acquisition and storage of samples and their appropriate management is essential for the reproducibility and biological relevance of subsequent qPCR results (Bustin, 2004). However, since differences in sample treatment are likely to affect results and reproducibility, it was clear from an early stage that it was important to report in detail where tissue samples were obtained, how they were processed and where and for how long they were stored, especially when considering gene expression analyses from tissue biopsies (Bustin, 2004). Since most researchers will already have gathered as much information as possible about their samples, the release of that information would not place an additional burden on the data gathering process for publication.

It is also necessary to provide details of sample processing reagents, since for example, the manufacturing process of polypropylene reagents used for microfuge tubes and micropipette tips can lead to the incorporation of contaminating chemicals that are able to cause denaturation of DNA fragments (Belostrerkovsii and Johnston, 1996) and cause DNA to adhere to the walls of microfuge tubes and pipette tips (Belotserkovskii and Johnston, 1997). Hence it is important to know what plastic ware has been used when troubleshooting discordant results.

The nucleic acid extraction process itself is another critical step and both DNA (Fridez and Coquoz, 1996) and RNA (Slater, 1988) extraction can be problematic,

especially as samples pass through numerous preparative steps prior to the qPCR assay, every one of which can introduce additional variability.

This has led to the proliferation of a huge number of different protocols, which have a significant impact on both quantity and quality of nucleic acid extracted (Fridez and Coquoz, 1996; Logemann et al., 1987; Chomczynski and Sacchi, 1987; Rupp and Locker, 1988; McCormick, 1989; Puissant and Houdebine, 1990; Jackson et al., 1990; Schneiderbauer et al., 1991; Longley et al., 1991; Shi and Liu, 1992; Rossen et al., 1992; Tsai and Olson, 1992; Cano et al., 1993; Kato et al., 1993; Foss et al., 1994; Wiedbrauk et al., 1995; Frank et al., 1996; Zhou et al., 1996; Monteiro et al., 1997; Su et al. 1997; Stewart et al., 1998; Fredericks and Relman, 1998; Stanta et al., 1998; Mahony et al., 1998; Masuda et al., 1999; Morrogh et al., 2007; Hammerle-Fickinger, 2010). An example of the remarkable lack of attention to detail in nucleic acid extraction procedures is a recent report that traces the poor quality of many RNA preparations extracted from tissue culture cells to the trypsinisation step, which is routinely used to dissociate adherent tissue culture cells prior to RNA extraction (Vrtacnik et al., 2014). Finally, the continued need to be aware of sample purification-dependent variability is illustrated by a recent comparison of exosome isolation protocols, which shows that the identification of exosome-specific functions and biomarkers is critically dependent on using appropriate, well implemented purification techniques (Van Deun et al., 2014).

Section 4

RNA quality

A publication from 2000 investigated the influence of a large number of analytical variables on the performance of RT-PCR assays carried out on clinical samples (Zippelius et al., 2000), including the effects of cDNA priming and choice of RTase. Characteristically, this publication neither mentioned nor assessed RNA quality, an omission that typifies a large number of qPCR-based publications to this day. It is not as though the importance of RNA quality was not known, since there was an early report commenting on the variability of RNA extraction (Rappolee, 1990) and around the first steps were being taken to develop more appropriate assays for the assessment of RNA (Swift et al., 2000; Sugita et al., 2001) and incorporate RNA integrity measures into models for quantifying RNA levels (Pfaffl, 2001). Yet despite continued emphasis on the need for quality assessment of RNA (Bustin, 2004; Bustin and Nolan, 2004; Bustin and Mueller, 2005; Nolan et al. 2006; Bustin and Mueller, 2006), a 2005 analysis of the practices of the research community found that 37% of laboratories did not quality assess their RNA, a finding that was “as unexpected as it is disconcerting” (Bustin, 2005) and a review felt the need to state that “There is an urgent need for universal agreement on basic issues such as quality and quantity control of RNA” (Bustin et al., 2005). Whilst by 2008 it was clear to many that it was unacceptable that most publications did not address the critical issue of RNA quality assessment (Bustin, 2008), a review discussing molecular medicine, gene-expression profiling and molecular diagnostics in terms of “putting the cart before the horse” had to concede that interest in RNA quality

was “distinguished by a prevalent lack of concern” (Bustin, 2008). Even as late as 2011 there was a need for a major publication to investigate the obvious “measurable impact of RNA quality on the variation of the reference genes, on the significance of differential expression of prognostic marker genes ... and on risk classification performance using a multigene signature” (Vermeulen et al., 2011).

RNA purity and consistency are additional important parameters that are easily overlooked. The use of spiked-in DNA or RNA to verify the absence of inhibitors has been recommended for a while (Nolan et al., 2006), but variable inhibition of different targets makes simple recommendations problematic (Huggett et al., 2008). However, it is essential to screen for inhibition of the RT and the qPCR reactions, especially when the aim is to identify differential expression profiles that vary by relative small amounts.

Section 5

Reverse Transcription

There was early recognition that different reverse transcriptases (RTases) have differing abilities to transcribe through the extensive secondary structure in mRNA (Buell et al., 1978; Brooks et al., 1995) and that consideration of mRNA structure is essential prior to any investigation into gene expression (Kuo et al., 1997). This led to the formulation of the concept of technical and biological variability as being important for the interpretation of RT-qPCR experiments in the context of clinical applications and the essential need to consider the variability of the RT step (Bustin and Dorudi, 1998). Subsequent reports showed that the method of cDNA priming affects both accuracy and reproducibility of RT-qPCR experiments (Zhang and Byrne, 1999; Lekanne et al., 2002) and that reactions primed by target-specific primers are linear over a wider range than similar reactions primed by random primers (Bustin and Nolan, 2004). The first unequivocal demonstration that high variability is an intrinsic property of the RT step was provided in 2004 with two hugely important, but woefully ignored publications. The first showed that RT-qPCR gene expression measurements are comparable only when the same priming strategy, reaction conditions and RNA concentration are used in all experiments (Stahlberg et al., 2004). The second reported that RT- dependent cDNA yields could differ by up to 100-fold and that this was significantly dependent on the choice of target gene (Stahlberg et al., 2004). This paper also made the equally important, but essentially disregarded suggestion that assays should be run at least as duplicates starting with the reverse transcription reaction,

advice augmented by a more recent publication (Tichopad et al., 2009). Yet another paper reported gene-related factors as the primary determinants of RT variability and called for an evaluation of the RFT robustness of control genes in RT-qPCR normalisation (Linden et al., 2012). The conclusions from these papers are rather stark and place a question mark behind many of the results reported in the scientific literature, especially with regards to the discovery and validity of RNA biomarkers in molecular medicine (Bustin et al., 2015). A recent report demonstrates a similar dependence of RT-dPCR accuracy on the choice of enzyme and target (Sanders et al., 2013).

Section 6

PCR efficiency

Good PCR amplification is exponential and in theory proceeds at 100% efficiency, but in practice not all PCR reactions display the same high efficiency. The practical importance of this fact was recognised early on, with the recognition that differences in amplification efficiencies between samples from leukaemia patients will result in the underestimation of residual disease in patient samples, with the weakest positive patient samples incurring the highest error (Meijerink et al., 2001). Many, if not most experiments targetting cellular mRNAs or miRNAs quantify changes in RNA levels across multiple samples relative to the levels of internal control RNA(s). This relative quantification has the advantage of not requiring standards with defined concentrations; however it does depend on the expression of the reference gene being relatively invariant and on the changes in target gene expression being relatively large. There are several algorithms that allow the calculation of relative expression levels, with the most widely used one known as the $2^{\Delta\Delta}$Cq method (Livak and Schmittgen, 2001), where Cq is the abbreviation for the quantification cycle, formerly known as Ct or Cp. This involves normalising the expression of the gene of interest (GOI) against an internal reference gene within a sample, followed by a comparison of the normalised difference in expression between two samples ($\Delta\Delta$Cq). Whilst calculations are straightforward, this method makes the critical assumption of constant doubling of amplicon and/or equal amplification efficiencies between amplification reactions and although the authors emphasise the need to establish the actual amplification efficiencies of each assay,

this continues to be ignored by the vast majority of researchers. Since actual efficiency can range from 60% to 100%, small differences between GOI and reference genes can lead to huge differences in relative expression ratios and generate distorted results.

An improved model for data analysis took into consideration the importance of incorporating qPCR efficiencies into any calculation of relative quantification involving a target and reference gene transcript (Pfaffl, 2001). It was followed by the development of a relative expression software tool (REST) based on PCR efficiencies that allowed the comparison of results for multiple target and reference genes between different sample groups (Pfaffl et al., 2002). These publications initiated a wide ranging discussion with regards to the appropriate calculation of PCR efficiency, a topic that remains under active investigation, discussion and widespread disagreement (Liu and Saint, 2002; Liu and Saint, 2002; Bar et al., 2003; Tichopad et al., 2003; Peirson et al., 2003; Rutledge and Cote, 2003; Ramakers et al., 2003; Nogva and Rudi, 2004; Rutledge, 2004; Nordgard et al., 2006; Schefe et al., 2006; Karlen et al., 2007; Rutledge et al., 2008; Rutledge and Stewart, 2008; Platts et al., 2008; Batsch et al., 2008; Yuan et al., 2008; Ruijter et al., 2009; Tuomi et al., 2010; Towe et al. 2010; Mallona et al., 2011).

The awareness of the need to validate raw quantification cycle data through further analyses has resulted in the development of numerous *in silico* tools (Bustin et al., 2011). These assist with qPCR efficiency correction, normalisation to multiple reference genes, treatment of replicates and error propagation as well as appropriate statistical tests, Many are freely available online, while others are bundled with qPCR instruments or are available from various software houses, with the best-known probably Genex (Bergkvist et al., 2010) and qBase Plus (Hellemans et al., 2007). The most comprehensive information for accessing and evaluating these programs is available at http://www.gene-quantification.de/main-bioinf.shtml, along with additional links to detailed reviews and other publications dealing with qPCR data analysis.

Section 7

Normalisation

The 2000 RT-qPCR review (Bustin, 2000), together with two follow-on reviews (Bustin et al., 2005; Bustin, 2002) and another publication (Tricarico et al., 2002) also pointed out the significant problems associated with the identification of a valid reference for data normalisation. It was clear that normalisation was a rather persistent problem and that none of the solutions proposed were ideal, but that the use of internal standards comprising single reference genes or rRNA was inappropriate, at least for studies involving tissue biopsies. An appropriate solution was only offered with the publication of the ground-breaking “geNorm” paper in 2002 (Vandesompele et al., 2002). Here the authors quantified the errors associated with the use of a single reference gene and developed a program that allowed the selection of a set of reference genes that showed the least variation in expression amongst all the samples. In addition, they identified the use of a single reference gene for accurate quantification as a major source of variability. Whilst geNorm was the first, it is by no means the only method for evaluating candidate reference genes. Between 2003 and 2006 a whole range of additional algorithms were published (Akilesh et al., 2003; Andersen et al., 2004; Pfaffl et al., 2004; Szabo et al., 2004; Haller et al., 2004; Brunner et al., 2004; Abruzzo et al., 2005; Huang et al., 2006), but none of them has achieved the wide usage of geNorm. It is also not obvious which of the many approaches is the most accurate one or helps generate the most reliable conclusions. It is however fair to say that any one of these methods is better than using a single, unvalidated reference gene to show

small fold-changes in relative expression of a target gene and then go on to claim the discovery of a new set of biomarkers.

The topic of normalisation and the consequences of using inappropriate reference genes continues to be widely discussed (Dheda et al., 2004; Huggett et al., 2005; Dheda et al., 2005; Kitchen et al., 2010; Marullo et al., 2010; Lanoix et al., 2012; Hellemans and Vandesompele, 2014), although a survey carried out in 2005 revealed that most researchers continued to use single, unvalidated reference genes (Bustin, 2005), a situation that has continued to the present day (Bustin, 2008; Bustin, 2008; Huggett and Bustin, 2011; Bustin et al., 2013; Dijkstra et al., 2014; Abdel Nour et al., 2014). The search for optimal normalisation procedures has also extended to the quantification of microRNA (Mestdagh et al., 2009; Sato et al., 2009), although even here the use of the most reliable method, using the global mean normalisation method that averages expression level of all expressed miRNAs may not be adequate for the purpose of comparing very different tissue types (Vandesompele and Mestdagh, 2014).

Section 8

Towards the MIQE guidelines

As far as I can ascertain, the first time a checklist was proposed for the purpose of improving the quality of RT-qPCR experiments was in 2002 (Bustin, 2002). These early, somewhat simplistic forerunners of the MIQE guidelines were as follows:

1. Strict validation of any reagent is required. The quality and quantity of RNA templates must be closely monitored and reported when publishing.
2. There must be standardisation of methodology for relatable comparison between laboratories.
3. Standard internal controls must be included to monitor RT and PCR efficiencies and results must be published.
4. Every RT-qPCR run should include an amplicon-specific standard curve generated by serial dilution of a sense strand oligonucleotide.
5. Reactions should be set up using robots to ensure minimal variability, and a consensus with regard to intra-sample variability must be established.
6. Normalisation of samples obtained from *in vitro* experiments can be carried out against a panel (probably two or three) of housekeeping genes whose expression has been shown to be unaffected by experimental conditions. This information must be included in any publication.
7. Normalisation of samples obtained *in vivo* must be carried out against cell number (if nucleated blood cells), rRNA or total RNA. There is no justification or reason for using housekeeping genes.

8. Any conclusions from experiments involving *in vivo* tissue samples are hindered by the heterogeneity of material, especially so when using tumour samples. The use of additional techniques, such as LCM, to enrich for individual cell types or cell location should become mandatory, as it is crucial for relevant data interpretation.

I was certainly not right when saying that reference genes should not be used when normalising *in vivo* samples, but this was stated before the concepts associated with Genorm were published. The other points remain generally valid today, and certainly the ideas promulgated here became generally recognised as important and worth considering.

A later review commented that “trust in the accuracy and integrity of the scientific literature is an essential prerequisite for maintaining scientific excellence and advancing knowledge. This calls for urgent action by researchers, reviewers and editors who need to agree a basic set of quality criteria and adhere to elementary procedures that result in the publication of reliable and reproducible data. Such a list must include delineating minimum quality standards for template preparation, validation and consistent use of cDNA priming methods, enzymes, protocols and, equally critically, appropriate analysis of data” (Bustin, 2008).

Section 9

The trigger for MIQE

The final spark that prompted a determined effort to develop guidelines for qPCR was my experience as an expert witness for the MMR vaccine litigation trial at the High Court of Justice in London and the US omnibus autism proceedings at the United States Court of Federal Claims.

In 1998 an association between gastrointestinal disease and developmental regression in a group of previously normal children was reported, with a commentary that in most cases, onset of symptoms was after MMR immunisation (Wakefield et al., 1998). Evidence presented in 2000 based on differential detection of measles virus (MeV) in autistic and control children using RT-qPCR and other methods was stated as being “compelling in relation to the presence of MeV in children with autistic enterocolitis” (O'Leary, 2000). In 2002 a paper described the use of a hydrolysis probe-based RT-qPCR assay to investigate the presence of persistent MeV in the intestinal tissue of children with a supposedly new form of developmental disorder and concluded that the data confirmed an association between the two (Uhlmann et al., 2002). The reported detection of measles virus in the intestinal tissue of autistic children was used to claim a link between the MMR vaccine and the development of a new, regressive form of autism in children. Probably as a direct effect of this controversy, MMR coverage in England fell from a peak of 92% in 1995 to 80% in 2003/04, and is still below 80% in parts of London (Map). In 2006, a 13-year old boy, who had not received the MMR vaccine, became the first person in the UK for 14 years to die of measles

A number of studies attempted to reproduce O'Leary's findings (Afzal et al., 2006; D'Souza et al., 2006; D'Souza et al., 2007). All failed to do so; instead they provided strong evidence for contamination being the cause of the positive findings. However, there were some technical differences between the original study and the three more recent ones in the choice of tissue (gut vs blood) or protocols (enzymes, real-time PCR chemistries). Therefore, whilst there was a strong suggestion that Prof O'Leary's laboratory was detecting contaminants, there was no proof.

In 2007, the first of approximately 4,800 cases in the US omnibus autism proceedings came to trial at the United States Court of Federal Claims, where all vaccine claims are managed and adjudicated by the congressionally created Office of Special Masters. This trial was designed to establish whether or not autism can be caused by the MMR vaccine and I presented evidence I had gathered during an exhaustive analysis of raw hydrolysis probe-based data underlying the results reported in 2002; a transcript from the autism omnibus proceedings is publicly available here. My analyses established a series of mistakes, inappropriate analysis methods and misinterpretations that could never have been gleaned from a study of the published paper. The main findings were:

1. Both RNA from fresh frozen (FF) and formalin fixed, paraffin-embedded tissue (FFPE) was used. Formalin fixation modifies and destroys RNA and makes it less accessible for amplification compared with RNA from fresh samples (Koch et al., 2006). However, whereas the difference in Cqs between control RNAs extracted from FF and FFPE was just over nine Cqs, the Cqs recorded for MeV F-gene RNA were approximately the same. Since any nucleic acid present during formalin fixation would have been affected in an identical manner, the obvious implication of these results is that the control target was present prior to formalin fixation, whereas the MeV F-gene target entered the sample after formalin fixation.

2. Samples with no RNA, as determined by the absence of amplification of a control RNA, nevertheless recorded MeV F-gene Cqs no different from samples that contained control RNA.

3. No or late amplification of control RNA was detected in samples where the RT step had been omitted. In contrast, the Cqs recorded for the MeV F-gene amplified without an RT step were in a similar range to most of the F-gene Cqs recorded from runs that included the RT step. Since MeV does not pass through a DNA stage during its life cycle, the assay was not detecting MV.

Despite the presentation of detailed evidence at the major autism trials in 2007 in the USA, three initial judgements and subsequent appeals judgements subsequently confirming that the underlying data were unreliable, as well as some exposure of the facts (Bustin, 2008; Bustin, 2013) there has been virtually no coverage of the serious errors made by the authors of that 2002 publication.

Section 10

MIQE

By 2009 it was abundantly clear that the 2002 O'Leary paper was not unique in using inappropriate methods or analyses and omitting adequate experimental detail. As a result, readers' ability to evaluate critically the quality of the results presented was seriously impaired and it was impossible to repeat published experiments. The particularly egregious use of qPCR in the MeV/MMR/autism context provided the final stimulus to initiate a push towards improving the technical aspects of qPCR assay design and reporting. The MIQE guidelines (Bustin et al., 2009) are the result of this consensus around the need to improve published information with relevant experimental detail to cover every aspect important to the qPCR assay itself, as well as issues relating to pre- and post-assay parameters. MIQE addresses the key areas of standardisation that define any qPCR experiment, namely study design, technical detail, analysis methods and statistics under a set of captions that describe a large number of individual elements: "Experimental design, sample, nucleic acids, reverse transcription, target, primers and probes, assay details, PCR cycling and data analysis". At first sight, these look daunting, arduous and over-exacting. In practice, it is clear that most, if not all of these parameters describe information that would be obtained as a matter of course during the experimental design, optimisation and validation stages. Importantly, there is a clear hierarchy with some parameters, labelled "E" (essential) in the published guidelines, indispensable for an adequate description of the qPCR assay, whereas other components, labelled

"D" (desirable) more peripheral, yet constituting an effective foundation for the realisation of best practice protocols.

An important corollary was that the guidelines provide a set of recommendations that could be used by journal reviewers not overly familiar with qPCR to evaluate the reliability of the experimental protocols and ensure the inclusion of all essential information in the final publication. The last two years have seen an increasing number of citations of the original publication in the peer-reviewed literature, which have now exceeded 3,000. The original publication has been followed by several editorials on their implementation (Bustin et al., 2010; Bustin and Penning, 2012; Bustin, 2013) and has encouraged the publication of MIQE guidelines for digital PCR (Huggett et al., 2013).

The most controversial aspects of the original MIQE guidelines related to the proposal that publications must divulge the sequences of any primers used and especially should also report the sequences of any probes. The rationale behind releasing the primer sequences was straightforward: an experiment cannot be reproduced if one of the principal reagents is unavailable. Lack of access to a probe sequence, on the other hand, does not preclude analysis of the specificity, efficiency and sensitivity of an assay; however, for completeness' sake it is but a small step to take for most researchers. Many commercial qPCR assays are not supplied with the primer/probe sequences, since most vendors consider this commercially sensitive information; usually there are also no details provided on empirical validation of each individual assay. Publications utilising such assays could not satisfy the original MIQE requirements, placing limits on a universal acceptance of MIQE. Consequently, an amendment of the original guidelines now requires either primer sequences or a clearly defined amplicon context sequence (Bustin et al., 2011).

Section 11

Outlook

Very belatedly, major scientific journals have come to realise that they have been seriously negligent in their approach to this subject and have started to address this issue (Anonymous, 2013; Anonymous, 2014; McNutt, 2014). These editorials announcing measures to “reduce irreproducibility” are a welcome, if long overdue acknowledgement of the inadequate reporting of experimental detail in much of the peer-reviewed literature. However, a recent survey of qPCR-based publication demonstrates very clearly that the problem addressed by the MIQE guidelines continue to persist (Bustin et al., 2013; Abdel Nour et al., 2014), with worrying implications (Dijkstra et al., 2014).

The MIQE guidelines have certainly had a major impact on qPCR practice, with all major instrument and reagent manufacturers training their field application specialists, actively supporting MIQE implementation by researchers and supporting and organising workshops around the world that publicise the advantages of conducting qPCR experiments that generate reliable and biologically or clinically meaningful results. Unfortunately, there are thousands of papers in the scientific literature that report results and conclusions that are unlikely to be correct and, despite the MIQE guidelines, thousands more are published every year (Garson et al. 2009). The consequences are clear: 85% (Prinz et al., 2011) to 95% (Begley and Ellis, 2012) of biomedical research is not independently reproducible and thousands of clinically irrelevant biomarkers are published every year with the result that 85% of research funding is wasted,

amounting to billions of dollar a year (Macleod et al., 2014). Retractions are on the increase (Steen, 2011; Steen, 2011; Wager and Williams, 2011) and there is a direct correlation between the impact factor of a journal and the number of retractions from that journal (Fang and Casadevall, 2011), interestingly mirroring the inverse correlation we noted between transparency and impact factor (Bustin, 2013). The question posed by David Horrobin in 2003 “Modern biomedical research: an internally self-consistent universe with little contact with medical reality?” (Horrobin, 2003) can now be extended to include the addition “and based on an abysmal standard of technical proficiency”. It will be a long time before the many contradictions apparent in every area of the life sciences are addressed, never mind corrected (Johnson et al, 2014). But who is going to lead this effort, and how many vested interests are there that will help keep a lid on this can of worms?

Section 12

Disclosure

I was an expert witness for the MMR vaccine litigation trial at the High Court of Justice in London and the US omnibus autism proceedings at the United States Court of Federal Claims. I was paid by the solicitors acting for the principal defendants SmithKline Beecham Plc, Smith Kline & French Laboratories Ltd, Merck & Co Inc, Sanofi Pasteur MSD Ltd and the US Department of Justice, respectively.

Section 13

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Chapter 2

MIQE compliant quantification strategies in RT-qPCR

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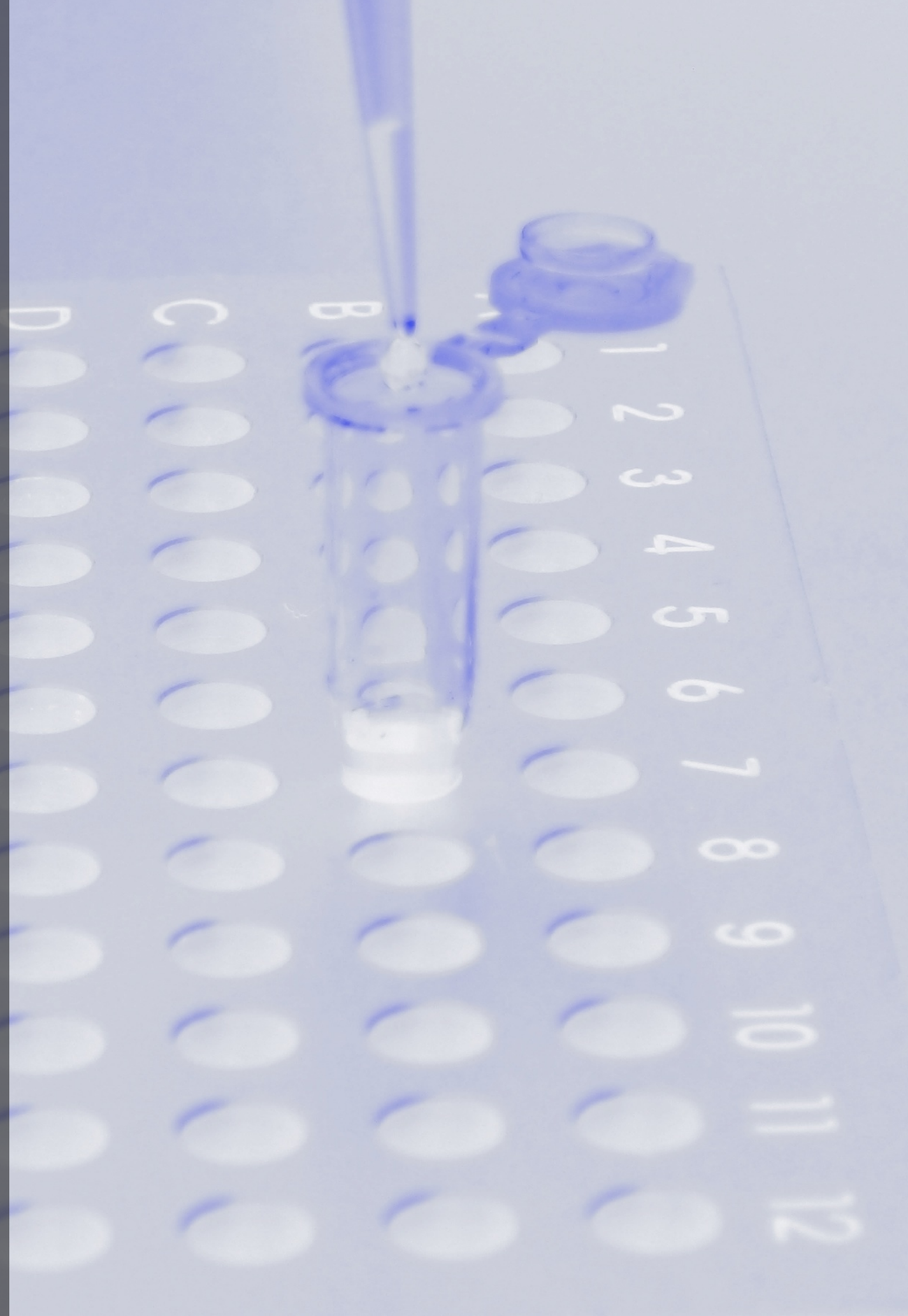

Section 1

Foreword

In this chapter, the two general methods for quantification of RNA using real-time RT-qPCR are described. Both absolute and relative quantification will be explained including requirements according to the MIQE guidelines for both qPCR and dPCR (Bustin et al., 2009; Huggett et al., 2013). The pros and cons of the quantification strategy types will be highlighted leading to a deeper insight into limitations, as well as how real-time PCR efficiency impacts the quantification result.

Section 2

Introduction

Reverse transcription (RT) followed by polymerase chain reaction (PCR) is the most commonly used technique for the detection and quantification of various RNA types. The first practical, kinetic PCR technology, the 5'-nuclease assay, was established in 1993 by Higuchi and co-workers by combining exponential PCR amplification with the monitoring of newly synthesized DNA in each PCR cycle (Higuchi et al., 1993; Heid et al., 1996; Gibson et al., 1996). In combination with RT, real-time RT-qPCR is a highly sensitive method for detection and quantification of messenger RNA (mRNA) transcripts, non-coding RNA or microbial RNA. Today, the fast and exact measurement of mRNA and microRNA (miR) by RT-qPCR is a critical element of life science research, molecular diagnostics and clinical applications. It represents the ‘gold standard’ for RNA quantification because of its excellent specificity, high sensitivity, good reproducibility and wide dynamic range of quantification (Orlando et al., 1998; Lockey et al., 1998; Morrison et al., 1998; Bustin, 2000). Low concentrations of RNA can be measured in small volumes, allowing gene expression measurements of low abundant transcripts down to 10 copies from limited tissue samples and even in single cells (Stahlberg and Bengtsson, 2010; Stahlberg et al., 2013; Stahlberg and Kubista 2014; Reiter et al., 2011).

While these PCR based methods show tremendous potential for use in any analytical or quantitative molecular diagnostic approach, a comprehensive understanding of its underlying principles and limitations is important (Pfaffl, 2004,

Remans et al., 2014). To provide guidance for researchers, reviewers and editors to measure the technical quality of the scientific work the MIQE guidelines were established for qPCR and dPCR (Bustin et al., 2009; Huggett et al., 2013). MIQE represents a set of guidelines that describe the minimum information necessary for evaluating qPCR or dPCR experiments and therefore set a clear framework from which to conduct validated and reliable quantitative PCR experiments. The goal is to support experimental transparency, promote consistent, comparable, valid and therefore reliable quantitative results.

The optimization and validation of the absolute or relative quantification strategies is one major point of the MIQE guidelines with the objective of increasing the credibility of results and helping to insure the integrity of scientific work, with major focus on biological relevance (Bustin et al., 2013; Dijkstra et al., 2014; Dooms et al., 2014; Sanders et al., 2011; Remans et al., 2014).

Link to media:

- MIQE summary
    - GeneQuantification.info
    - Miqe.info
- MIQE press review
- MIQE Guidelines: A Roadmap for Proper qPCR Experimental Design and Reporting

Section 3

Quantification strategies

The decision regarding which RT-qPCR quantification strategy is chosen is a critical component of the gene quantification workflow and a required declaration under the MIQE guidelines. Generally, two strategies can be performed in quantitative RT-PCR. The concentrations of expressed genes may be measured by absolute or relative quantification.

Absolute quantification relates the PCR data to input copy number using a calibration (or standard) curve. Therefore, this calibration curve is used to determine the concentration of the unknown analyte. Although traditionally referred to as ‘absolute quantification’ it is preferable for this approach to be called ‘calibration quantification’. This is because, in this context, the word ‘absolute’ is misleading, as the concentration of the field sample in fact is measured ‘relative’ to the concentrations of the standard samples used in the calibration curve (Svec et al., 2015). Furthermore, the reliability of an absolute real-time RT-PCR assay depends on the condition of ‘identical’ amplification efficiencies for both the native target and the calibration curve in both the RT reaction and in the following qPCR (Souaze et al., 1996; Pfaffl, 2001; Pfaffl et al., 2002).

Relative (sometimes referred to as ‘comparative’) quantification does not need a calibration curve. For this reason it would initially appear that these experiments are easier to perform than absolute quantification. Relative quantification is used to measure the change in mRNA expression relative to chosen control samples (Livak and Schmittgen, 2001). It is mainly based on the expression of a target gene

versus one or more endogenously expressed reference genes (Vandesompele et al., 2002), originally and commonly known as 'housekeeping genes'. The relative quantification approach is adequate for most purposes, such as to investigate physiological changes in gene expression for example when measuring mRNA or microRNA gene expression changes. The unit used to express relative quantities is commonly expressed as 'x-fold change' and allows the relative quantities can be compared across multiple experiments (Orlando et al., 1996; Livak and Schmittgen, 2001).

Recently digital PCR (dPCR) became more attractive as a measure of absolute quantification (Kalinina et al., 1997; Vogelstein and Kinzler, 1999). Using dPCR, single molecules are separated by dilution and are amplified individually in defined units or partitions. After amplification each partition is analysed and represents a single starting molecule. Digital PCR transforms the exponential, analogue classical PCR amplification into a linear and digital signal (Morley 2014; Sanders et al., 2011). An overview of the MIQE compliant quantification strategies is shown in Figure 2.1.

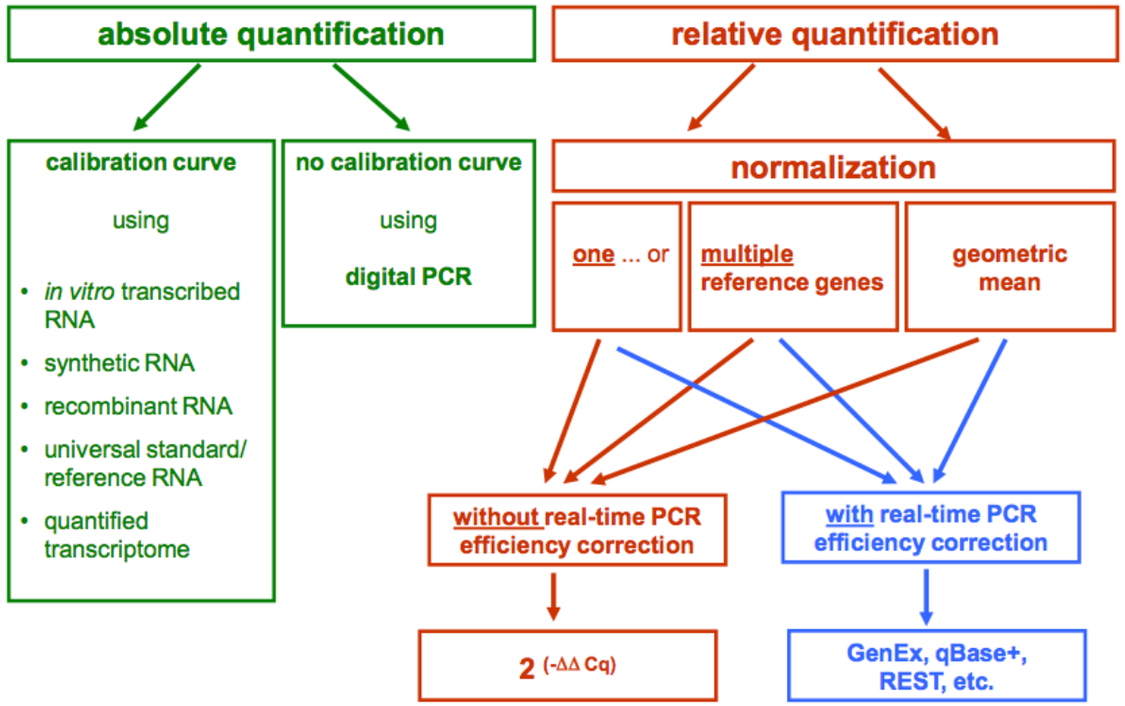

Figure 2.1 - MIQE compliant strategies in RT-qPCR

Link to media:

- Summary about quantification strategies
- TATAA tutorials: absolute quantification, relative quantification, and a lot more -

Section 4

Absolute quantification

In order to apply the absolute quantification approach, a stable standard RNA material is required. Since this material is used to generate the calibration curve, it must have a known concentration. When correctly applied, the calibration curve is highly reproducible and allows the generation of highly specific, sensitive and reproducible data (Bustin, 2000; Pfaffl et al., 2002; Rutledge and Côté, 2003; Svec et al., 2015). The external calibration curve model has to be thoroughly validated, as the accuracy, sensitivity, reproducibility and the range of quantification depends entirely on the accuracy of the standards used. However, there are several difficulties associated with production of an RNA standard. The standard RNA design, synthesis, determination of exact concentration and assured stability over long storage times is not straightforward. At low concentrations, RNA is highly sensitive to degradation and therefore, needs storing in a good buffer, an RNAse free environment and responsible handling by the researcher. The stability of RNA standard material of low concentration (e.g. lower than 100 molecules) is particularly critical for high reproducibility (Reiter et al., 2011). As shown in Figure 2.2, at low ($< 10^3$) template copy input the assay variability is higher than in the range of higher copy number (Pfaffl and Hageleit, 2001; Rasmussen, 2001; Sanders et al., 2014). At very low copy numbers, under 20 copies per tube, the random variation due to sampling error (Poisson's error law) becomes significant (Peccoud and Jacob, 1996; Rasmussen, 2001). But it is exactly in this variable region that target quantification is of high interest for molecular diagnostics, e.g. to

detect RNA viruses or bacteria at low plasma concentrations, early after infection, or to quantify rare transcripts in limited sample material.

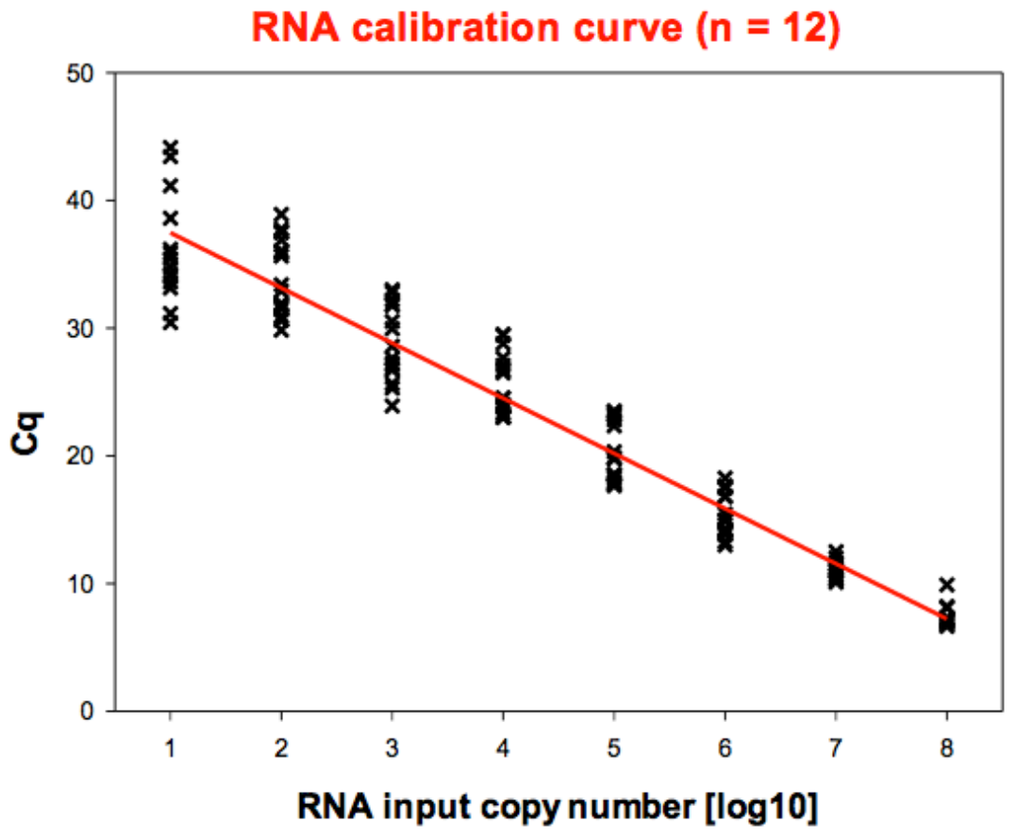

Figure 2.2 - RNA Standrad Curve

The dynamic range of the calibration curve can be up to seven orders of magnitude from 10^1 to 10^8 standard molecules, depending on the stability of the used RNA standard material (Pfaffl and Hageleit, 2001; Fronhoffs et al., 2002; Reiter et al., 2011). It is recommended that the calibration curve should have at least five, 10-fold dilutions, for each three technical replicates (Svec et al., 2015). The stability and reproducibility depends on the type of standard material used, the surrounding matrix/tissue extract, the presence of RNAse and the general operator handling according to 'good laboratory practice'. For suitable RNA standard material, commercially synthesized RNA may be used, or alternatively in vitro transcribed RNA, recombinant RNA, universal standard/reference material or alternatively a transcriptome with known concentration of the mRNA or microRNA transcript of interest (Pfaffl and Hageleit, 2001; Cronin et al., 2004).

A significant problem with RNA based calibration curves is that they are subject to the variability of the reverse transcription reaction (RT), which increases the variability of the calibration curve especially at low concentrations (Reiter et al., 2011; Bustin et al, 2014). To deliver the best possible, most reproducible results, the RT should be performed in parallel on the biological sample and the calibration curve. It has been demonstrated that the RT reaction is highly dependent on the RT enzyme used (from <3% to over 70% RT efficiency) and shows high variability between technical replicates (up to 30%) (Stahlberg et al., 2004a and 2004b; Bustin et al, 2014). The goal is to derive identical RT efficiency in all biological samples performed, as well as real-time PCR amplification efficiency for calibration curve standard RNA and target RNA. This must be validated and confirmed in advance, to receive later reliable RNA quantification results.

A further significant problem when using an RNA quantification procedure is due to the impact of RNA secondary structure. Natural occurring cloverleaf structures of long RNAs (e.g. mRNAs or primary microRNAs of around 2kb) and buffer or pH related secondary folding have an impact on RNA sequence accessibility. Artificial produced RNA standard material may have different folding properties when compared to native, isolated RNA fractions due to varying length, stabilizing matrix, or buffer substances. Furthermore, differently folded RNA will influence the accessibility of endo- and exo-RNAse and thus consequent resistance to degradation, as well as the primer binding and the resulting RT efficiency due to enzyme processivity (Stahlberg et al., 2004a; Stahlberg et al., 2004b; Bustin et al, 2014).

In contrast to naturally occurring RNA, isolated from biological samples, artificial RNA standard material is highly homogeneous, uniform in length and sequence motives. Natural biological samples contain a high percentage of sub-fractions, e.g., ribosomal RNA (rRNA 85-90%), transfer RNA (tRNA ~ 5-10%), messenger RNA (mRNA ~ 1-5%) and various families of non-coding RNAs e.g. microRNA, piRNA, siRNA, snRNA, snoRNA, long non-coding RNAs (~ 1-5%) (Kiss, 2001). Varying RNA sub-fractions have an influence on the cDNA synthesis rate and consequently on the validity of the calibration curves but in an unknown manner (Pfaffl, 2004). To compensate for such complex RNA background effects, a natural RNA distribution can be mimiced with a spike-in RNA isolated from a diverse organism to that of study such as plant, bacterial or insect cell lines when human samples are being investigated. Alternatively, commercially available RNA spike-in sources can be used as RNA background, e.g., poly-A RNA or tRNA. However, these do not represent a native RNA distribution over all RNA subtypes. Earlier published results suggest that a minimum of RNA background is generally needed and that it enhances RT synthesis efficiency rate. Low concentrations of standard RNA used in calibration curves should always be buffered with background or carrier RNA; otherwise the low amounts can be degraded easily by RNAses or be bound to reaction vessels. Very high background concentrations have a more significant suppression effect in RT synthesis rate and in later real-time PCR efficiency (Tichopad et al., 2009; Stahlberg et al., 2004b; Bar et al., 2012)

The sensitivity problem of the RT-PCR to minor variations in the reaction setup is always lurking in the background as a potential drawback to this simple procedure. Therefore, ‘absolute quantification’ with external RNA standards requires careful, time consuming optimization of its precision (by using technical replicates in the same PCR run, reported as intra-assay variation) and reproducibility (using technical replicates in separate PCR runs, reported inter-assay variation) in order to understand the limitations within the given application and fulfil the MIQE guidelines (Pfaffl and Hageleit, 2001; Reiter et al., 2011).

No matter how accurately the concentration of the used standard material was determined, the final quantification result is always reported on the basis of a user friendly unit, e.g. RNA copies per ng transcriptome (total RNA), copies per isolated cell, copies per mg of starting matrix or tissue, copies per ml blood, etc. If absolute changes in copy number are important (copy number variations), then the denominator must still be shown to be absolutely stable across the compared biological samples. This accuracy may only be needed in immunology and microbiology, to measure the viral load in human tissues, or in food hygiene to measure the number of viruses or microorganisms present in edible matrices. The determination of absolute quantity of your setup cannot be better than the quantity of the denominator. Any variation in the denominator will obscure real changes, produce artificial changes and incorrect absolute quantification results. The careful use of standard material and controls is critical in order to demonstrate that your choice of denominator is appropriate (Rasmussen, 2001).

Link to media:

- Absolute quantification summary
- TATAA Biocenter / Clontech online seminar
- Sigma Aldrich online seminar
- Bio-Rad Real online tutorial – real time qPCR data analysis tutorial

Section 5

Absolute quantification via dPCR

Digital PCR (dPCR) is a currently developed PCR based method for the absolute quantification of nucleic acids. It allows precise quantification, facilitating the measurement of small percentage differences or the quantification of rare variants (Vogelstein and Kinzler, 1999; Pabinger et al., 2014). Applying dPCR for RNA analysis it suffers from the same problems described earlier, with the major reproducibility problems in the RT step. But various papers have demonstrated that dPCR may also be more reproducible and less susceptible to inhibition than classical quantitative RT-qPCR (Sanders et al., 2013).

The technical details of dPCR are described extensively elsewhere in this MIQE & qPCR b book ook (Chapter Applying MIQE standards to dPCR experiments). To summarise the approach, the PCR reaction mixture is divided into a very large number of separate, tiny volumes. The goal is to achieve a maximal dilution, such that there is either zero or one target molecule present in any individual reaction unit. Currently available commercial systems generate multiple tens of thousands of droplets (Bio-Rad, Fluidigm, Life Technologies) or even up to ten million droplets per experiment (RainDance) (Baker, 2012; Pabinger et al., 2014). After performing PCR amplification, the reporter signal output is counted for each reaction vessel/ droplet such that the reaction results then becomes equivalent to a digital output (in that a positive reporter signal is equivalent to one target molecule per reaction vs. no reporter signal which indicates no target molecule). After applying a ‘Poisson correction’ to account for wells which may contain more than one copy,

the reporter signal counts are used to quantify the absolute molecule number present in the test sample. This absolute quantification is based on ‘simple counting’ and needs no calibration curve. It has been reported to have a higher accuracy that qPCR (Kalinina et al., 1997; White et al., 2009). Recently, a study systematically compared the performance of qPCR with that of dPCR. The authors could clearly demonstrate that droplet based dPCR exhibited greater precision and more reproducible findings than qPCR (Hindson et al., 2013; Marx, 2014).

Link to media:

- Digital PCR summary
- Summary of digital PCR presentations

Section 6

Relative quantification

Relative quantification is typically used to determine the changes in steady-state RNA concentrations of an expressed gene of interest across multiple samples. The results are expressed relative to the concentrations of one or more endogenous control RNAs. This approach is adequate for investigating the physiological changes in mRNA or microRNA gene expression between test or treatment groups. Therefore, relative quantification does not require an obligatory calibration curve consisting of RNA standards with known concentrations. Reporting biologically meaningful changes in mRNA copy number requires accurate and relevant normalization to some standard gene expression and is strongly recommended according to the MIQE guidelines (Bustin, 2002; Bustin et al., 2009). The selected reference gene transcript is often a ‘housekeeping gene’, an artificial RNA sequence or can be any transcript, as long as its sequence is known. (Bustin, 2002; Gilsbach et al., 2006). Dependent on the assay type, the reference genes can be co-amplified with the target gene in the same tube in a multiplexed assay or can be amplified in a separate tube (singleplex assay). Ideally, the expression of reference gene(s) should remain unchanged across samples within the context under study (Wittwer et al., 2001). The reliability of the relative RT-qPCR experiment can be even improved by including an invariant ‘exogenous and artificial control RNA’ to correct for sample-to-sample variations in RNA extraction, RT efficiency, or errors in sample quantification workflow.

A further method is described for the relative comparison of two RNA amplicons, the 'Relative Standard Curve Method', by using two 'absolute' quantification approaches. Multiple transcripts are quantified separately according to the described classical calibration curve and the resulting 'absolute quantities' are compared relatively (Cikos et al., 2003).

More details and information about normalisation strategies are shown in Chapter: Choosing Reference genes.

Link to media:

- Relative quantification summary
- Presentation - From reference genes to global mean normalization
- Presentation - RefGenes
- Presentation - qBase+

Section 7

Reference gene selection

The choice of suitable single reference gene or the selection of the appropriate panel of reference genes is absolutely crucial for accurate RT-qPCR gene expression analysis and is a required reporting element of the MIQE guidelines (Bustin et al., 2009). There are various, commercially available reference gene panels to select from (e.g. Life Technologies, Exiqon, TATAA Biocenter, PrimerDesign, etc. summarized in Vandesompele et al. 2009), but unfortunately these may not work well for the specific biological context, for example if the focus of study is an organism outside the usual human, mouse or rat. Depending on the study and the tissue under investigation, pre-selected reference gene panels may vary significantly and do not exhibit the optimal characteristics of a feasible and stable reference gene.

In the published literature, there is an accumulation of applied reference genes, e.g., GAPDH, albumin, actins, tubulins, cyclophilin, microglobulins, 18S rRNA or 28S rRNA. Unfortunately, these are often applied without verification and independent of consideration of the physiological or biological context (Foss et al., 1998; Thellin et al., 1999; Goidin et al., 2001; Vandesompele et al., 2002; Laule et al., 2006; Hruz et al., 2011). All this candidate genes belong to the group of ‘housekeeping genes’ and are theoretically optimally suited, since they are present in all nucleated cell types and are necessary for innate cell survival. The mRNA synthesis of these ‘housekeepers’ is considered to be high abundant and stable expressed in various tissues, even under experimental treatments. However,

numerous treatments and studies from more than 20 years ago have conclusively shown that these targets are regulated and that they do vary significantly under many experimental conditions (Tichopad et al., 2003 and 2004). It remains up to the individual investigator to choose a reference gene set that is 'best suited' for reliable normalization in their particular experimental and biological context.

Therefore a prior selection of adequate reference gene candidates is critical and an absolute experimental requirement. Recently an algorithm was established, named RefGenes, to identify reliable and context specific reference genes for RT-qPCR data normalization (Hruz et al., 2011). The RefGenes algorithm is part of the Genevestigator database of normalized and well-annotated microarray experiments (Laule et al., 2006; Hruz et al., 2008), and describes the expression stability characteristics of the transciptomes of several tissues and organisms (to date 17 species, >1400 cell lines, >120.000 samples). Analyzing numerous biological experiments, it provides the confirmation that specific genes are neither universally nor stable expressed in varying biological contexts. Further it demonstrates that the most commonly used reference gene candidates show very high transcript abundances as compared to the entire transcriptome. For each biological context a subset of stable expressed genes exists that has smaller variance than commonly or commercially used reference gene panels or genes that were selected for their stability across all conditions. Therefore, when possible, reference genes may be specifically chosen using the RefGenes algorithm for the particular condition(s) under study.

Link to media:

- Genevestigator Video tutorial
- RefGenes Video tutorial
- Sigma Aldrich Seminar - Reference Gene Validation

Due to the request of the MIQE guidelines, there is an increasing appreciation of the correct processes for data normalisation using valid normalisation algorithms. It is now well accepted that more than one reference genes should usually be used for normalisation, since averaged multiple reference genes result in better stability for normalisation, for mRNA as well as for microRNA profiling. It is obvious and proven, that no single gene is constitutively expressed in all cell types and under all experimental conditions. Therefore the expression stability of the intended reference genes has to be verified separately for each experimental setup. For large scale expression profiling studies with a lot of candidate genes under investigation, normalization using a global mean expression value or a reference residual normalisation (RRN) may outperform the general normalization strategy based on a 'few' validated reference genes (Mestdagh et al., 2009; D'haene et al., 2012; Edmunds et al., 2014). Suitable algorithms for reference gene selection include, geNorm (Vandesompele et al., 2002), BestKeeper (Pfaffl et al., 2004),

NormFinder (Andersen et al., 2004) and Pattern-Recognition-Analysis (Akilesh et al., 2003).

• The tool with the widest worldwide distribution is probably geNorm, which allows for an accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple, internal control genes (Vandesompele et al., 2002). The geNorm algorithm determines the most stable reference genes from a given gene set and calculates a 'gene expression normalization factor' for each biological sample based on the geometric mean of a user defined number of reference genes.

Link to geNorm : http://medgen.ugent.be/~jvdesomp/genorm/

• BestKeeper determines the best suited reference genes, out of ten reference candidates, and combines them into an index, the 'housekeeping gene index' (Pfaffl et al., 2004). The software predicts the 'optimal' reference genes, by employing the pair-wise correlation analysis of all pairs of candidate genes and calculates the geometric mean of the 'best' suited ones. In this way, the BestKeeper Index (or reference gene index, RGI) is calculated, which is based on the weighted expression of at least three stable reference genes and a more reliable basis of normalization in relative quantification can be postulated.

Link to Best Keeper : http://bestkeeeper.gene-quantification.info

• The NormFinder algorithm represents a further, robust strategy to identify stably expressed reference genes from a set of candidate normalization genes (Andersen et al., 2004). The strategy is rooted in a mathematical algorithm of gene expression that enables the estimation of the overall suitability of any normalization gene candidate in any kind of experimental design. NormFinder allows a more reliable normalization of gene expression data variation of the candidate reference genes and also of the variation between sample subgroups of the biological sample set. Notably, the strategy provides a direct measure for the estimated expression variation, enabling the user to evaluate the systematic error introduced when using the chose reference gene.

Link to NormFinder : http://moma.dk/normfinder-software

Application of any of these three normalization strategies is a prerequisite for accurate relative quantification and expression profiling using RT-qPCR and is recommended by the MIQE guidelines.

Section 8

Normalization of RT-qPCR data

Many different methods have been proposed for accurate normalization of RT-qPCR data (Huggett et al., 2005). To calculate the expression of a target gene in relation to an adequate reference gene, various mathematical models have been established and implemented in software tools, e.g. GenEx (Stahlberg et al., 2008; Bergkvist et al., 2010), qBase+ (Hellemans et al., 2007), REST (Pfaffl et al., 2002), as well as more applets that are integrated into real-time cycler data analysis software.

The general calculation procedures are based on the relative comparison of the distinct quantification cycle used for the quantification procedure. According to the MIQE guidelines and RDML (Real-Time PCR Data Markup Language) (Lefever et al., 2009) the new term ‘quantification cycle’ (Cq) was introduced as a substitute for the range of previously used nomenclature such as ‘crossing point’ (CP) and ‘threshold cycle’ (Ct) or ‘take off point’ (TOP).

Several mathematical models that determine the relative expression ratio have been developed. These can be grouped in two general types (see Figure 1):

- relative quantification without PCR efficiency correction, assuming optimal qPCR efficiency of 100% or 2.0 (Livak and Schmittgen, 2001)
- relative quantification applying correction to accommodate the actual PCR efficiency (Pfaffl, 2001)

When using the (Livak and Schmittgen, 2001) approach, the relative expression change of a target gene of interest is computed following these steps:

1. Determination of the Cq difference (Cq) between Cq ‘gene of interest’ (GOI) and Cq ‘reference gene’ (REF) for each sample
2. The PCR efficiency is assumed to be 2.0, according to the optimal doubling of PCR products in each cycle performed (Livak and Schmittgen, 2001);
3. Determination of the differences between the calculated Cq (from step 1) for the ‘treatment group’ and ‘control group’. This results in a Cq value.
4. The relative expression ratio (R) represents the ‘x-fold regulation’ of one GOI undergoing the applied treatment in comparison to the untreated control group, normalized to the stable expression of one REF, assuming the optimal PCR efficiency of 2.0 for all genes under calculation.

$$\Delta Cq = Cq_{GOI} - Cq_{reference\ gene}$$

$$R = 2^{-[\Delta Cq_{treatment} - \Delta Cq_{control}]}$$

$$R = 2^{-\Delta\Delta Cq}$$

Section 9

Real-Time PCR amplification efficiency

Each qPCR amplification history is unique. Individual real-time RT-qPCR reaction setups generate differing fluorescent amplification plots, in which can be described according to the shape of the background, exponential and plateau phase. The integrity of an optimal qPCR run can be described by these phases, by a low and stable background phase, the initiation of an increase in fluorescence (correlates with Cq), steepness of the fluorescence increase (correlates with PCR efficiency), and the absolute fluorescence levels at the plateau (correlates with the amount of PCR products). The PCR amplification efficiency has a major impact on this fluorescence history and therefore on the RT-qPCR performance. The PCR efficiency influences the entire amplification process through all of the performed cycles. Hence, PCR efficiency evaluation is one of the essential markers of the real-time gene quantification procedure and a core characteristic that is required by the MIQE guidelines.

PCR efficiency, and by implication, the accuracy of the calculated expression result, is influenced by various variables, as shown in Figure 2.3. It is well known that different tissue types may impact RT and PCR efficiencies, caused by RT or PCR inhibitors and by variations in the total RNA fraction pattern extracted (Tichopad et al., 2003 and 2004). Stable and constant amplification efficiency in all compared samples is one important criterion for reliable comparison between samples. This becomes crucially important when analyzing the relationship between biological samples with unknown characteristics, as compared to

controls. A PCR efficiency correction is strongly recommended and results in a more reliable estimation of the 'true expression ratio' when compared to no-efficiency correction (Pfaffl, 2001).

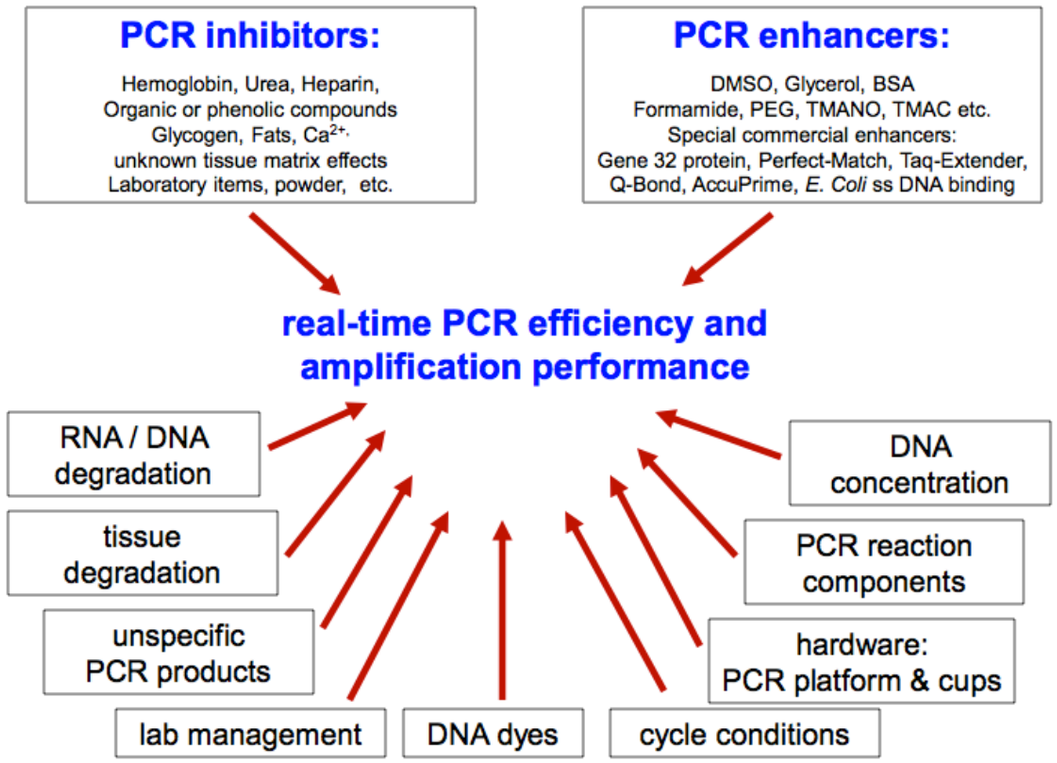

Figure 2.3 - Factors impacting PCR efficiency

The assessment of reliable PCR amplification efficiencies of all transcripts under investigation; all gene of interests and reference genes, must be carried out before any calculation of the normalized relative gene expression is performed. Several methods have been described to calculate real-time PCR efficiency and these are implemented in qPCR data analysis tools, e.g. GenEx (Stahlberg et al., 2008; Bergkvist et al., 2010), qBase+ (Hellemans et al., 2007), Q-Gene, (Muller et al., 2002), REST (Pfaffl et al., 2002), LightCycler Relative Expression Software (Roche Molecular Biochemicals, version 1.0, 2001), or open access software packages allow the evaluation of amplification efficiency plots (summarized in Pabinger et al., 2014).

Link to media:

- PCR efficiency summary
- A survey of tools for the analysis of qPCR data

Methods to determine real-time PCR efficiency:

• Serial dilution approach to determine a 'mean' PCR efficiency: Commonly a serial dilution is used to calculate the mean PCR efficiency (Figure 2.4). The efficiency is calculated from the slope of the dilution series using the equation: $E = 10^{[1-slope]}$ This results in values ranging from E = 1.0 (minimum value) to E = 2.0 (theoretical maximum and optimum efficiency) (Higuchi et al., 1993; Rasmussen, 2001, Livak & Schmittgen, 2001). Determination of PCR efficiency should be evaluated in a pool of all starting RNAs to accumulate all possible 'positive' and 'negative' impacts on efficiency. Typically, the relationship between Cq and the logarithm of the starting copy number of the target sequence should remain linear for at least five orders of magnitude in the calibration curve as well as in the native RNA sample. A separate determination of real-time PCR efficiency can be made using triplicates for every transcript and target tissue. This is necessary to determine it with high precision and validity (Souaze et al., 1996; Pfaffl and Hageleit, 2001; Svec et al., 2015). Optimally the values are 2.0±0.1 (Life Technologies Application Note, 2012). Unfortunately the 'dilution series method' sometimes results in efficiencies far higher than 2.1, which is practically impossible according to the PCR amplification theory. But as shown by numerous publications, the results are constant and highly reproducible, at least within one transcript and tissue (Pfaffl et al., 2001).

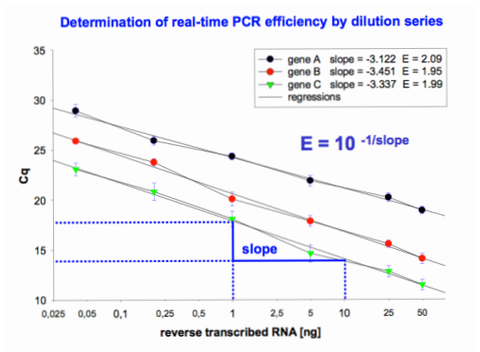

Figure 2.4 – Dilution Series for Determination of qPCR Amplification Efficiency

• Single run PCR efficiency determination based on all cycle data: A more advanced method is to determine the efficiency on the basis of a complex modelling of the fluorescence history data to sigmoidal or logistic algorithms. It can be applied to single reaction setups and therefore each investigated biological sample can be analyzed individually. The advantage of this model is that all fluorescence history data points are included in the calculation process and no background subtraction is necessary (Liu & Saint, 2002a and 2002b; Tichopad et al., 2003). Unfortunately, even for optimal reaction setup, the derived efficiency values generated after applying mathematical modelling algorithms range between 1.7 and 1.9 and so are not directly comparable to those from the 'dilution series'. This difference might be due to the sigmoidal or logistic fitting procedure and could reflect a single biological sample rather than an average estimate between various replicates and dilutions. The big advantage is that these advanced fitting algorithms are fully automatable in the cycler data analysis software (e.g. RotorGene, Eppendorf, LightCycler and more) and therefore easy to perform.

• Single run PCR efficiency determination based on selected cycle data: Applying this approach only selected data from the exponential amplification phase is integrated into the PCR

efficiency determination. At the exponential phase of the PCR reaction, the reaction kinetics is still under ‘full amplification power’ with no resource limitations. The calculation is performed on each reaction kinetic plot and the maximal amplification efficiency can be determined. The calculation is based on the fluorescence increase only in the real exponential phase, according to a polynomial curve fit $Y_n = Y_0 (E)^n$, where Y_n is the fluorescence acquired at cycle n and Y_0 the initial fluorescence, or ground fluorescence (Bar et al., 2012; Tichopad et al., 2003; Ramakers et al., 2003). This phase around the ‘second derivate maximum’ exhibits real exponential amplification behaviour (LightCycler Software, 2001). Calculated PCR efficiencies of reactions under optimal conditions range from E = 1.75 to E = 1.90, hence these lie between those derived using the previously mentioned methods.

Today the ‘dilution series’ is the most applied for PCR efficiency determination and suits the recommendations by the MIQE guidelines (Bustin et al., 2009). The both single run methods need deeper understanding in data collection and curve fitting, otherwise it is not calculated automatically by the real-time cycler software. Which efficiency calculation method is ‘the right’ one and results in ‘true PCR efficiency’ results has to be evaluated in further methodological experiments.

Section 10

Calculating Gene Expression Fold Changes using Efficiency Correction

Since PCR efficiencies vary between genes, assays, samples, reagents and instruments, introducing a real-time PCR efficiency (E) correction helps to provide a more accurate relative quantification (Pfaffl, 2001). The relative fold change is then defined as:

$$R = \frac{(E_{REF})^{Cq\ treatment}}{(E_{GOI})^{Cq\ treatment}} \div \frac{(E_{REF})^{Cq\ control}}{(E_{GOI})^{Cq\ control}}$$

Since PCR efficiency differences usually exist within the applied PCR assays, e.g. gene by gene, the formula can be expressed as:

$$R = \frac{(E_{GOI})^{\Delta Cq_{GOI}(control-treatment)}}{(E_{REF})^{\Delta Cq_{REF}(control-treatment)}}$$

In this case the calculation steps are:

1. Determination of the Cq difference (Cq) for the ‘gene of interest’ (GOI) between the ‘control sample’ and the ‘test sample’
2. Determination of the Cq difference (Cq) for the ‘reference gene’ (REF) between the ‘control sample’ and the ‘test sample’
3. Determine the PCR efficiency for each gene (GOI and REF)
4. The relative expression ratio (R) represents the ‘x-fold regulation’ of one GOI undergoing the applied treatment in comparison to the untreated control group, normalized to the stable expression of one REF, assuming the optimal PCR efficiency of E for the REF or GOI genes under calculation.

Applying the ‘Relative Expression Software Tool’ (REST) the relative calculation procedure is based on an averaged Cq (MEAN Cq) for the experimental groups, either treatment or control group, and the averaged normalisation of multiple reference genes, the ‘reference gene index’ (RGI) (Pfaffl et al. 2002).

$$R = \frac{(E_{GOI})^{\Delta Cq_{GOI}(MEAN\ control - MEAN\ treatment)}}{(E_{RGI})^{\Delta Cq_{REF}(MEAN\ control - MEAN\ treatment)}}$$

If relative quantification carried out according to the MIQE guidelines, applying PCR efficiency correction and using multiple validated reference genes, the data generated are more likely to represent the true biological status of the tested genes at the time of sampling (Pfaffl, 2004; Bustin et al., 2009).

Link to media:

- Summary of qPCR normalisation
- Relative Expression Software Tool http://REST.gene-quantification.info/

Section 11

Conclusion

The quantification strategy used for a reliable mRNA or microRNA transcript measurement should be designed according to the biological question. To be authentic, trustworthy and MIQE compliant, the applied quantification strategies must be highly optimized and precisely validated. In the recent years numerous software tools have been released which standardise the quantification process and make the results more reproducible (summarized and discussed in Pabinger et al., 2014). The ultimate aim is to decrease the intra-lab variability between runs, assays and applied SOPs. Also the standardisation and comparability between real-time RT-PCR platforms, analysis software tools, and between different laboratories worldwide is favoured. Therefore sharing protocols, technical and practical achievements and improvements will help to improve gene quantification workflow and spread the MIQE concept.

Section 12

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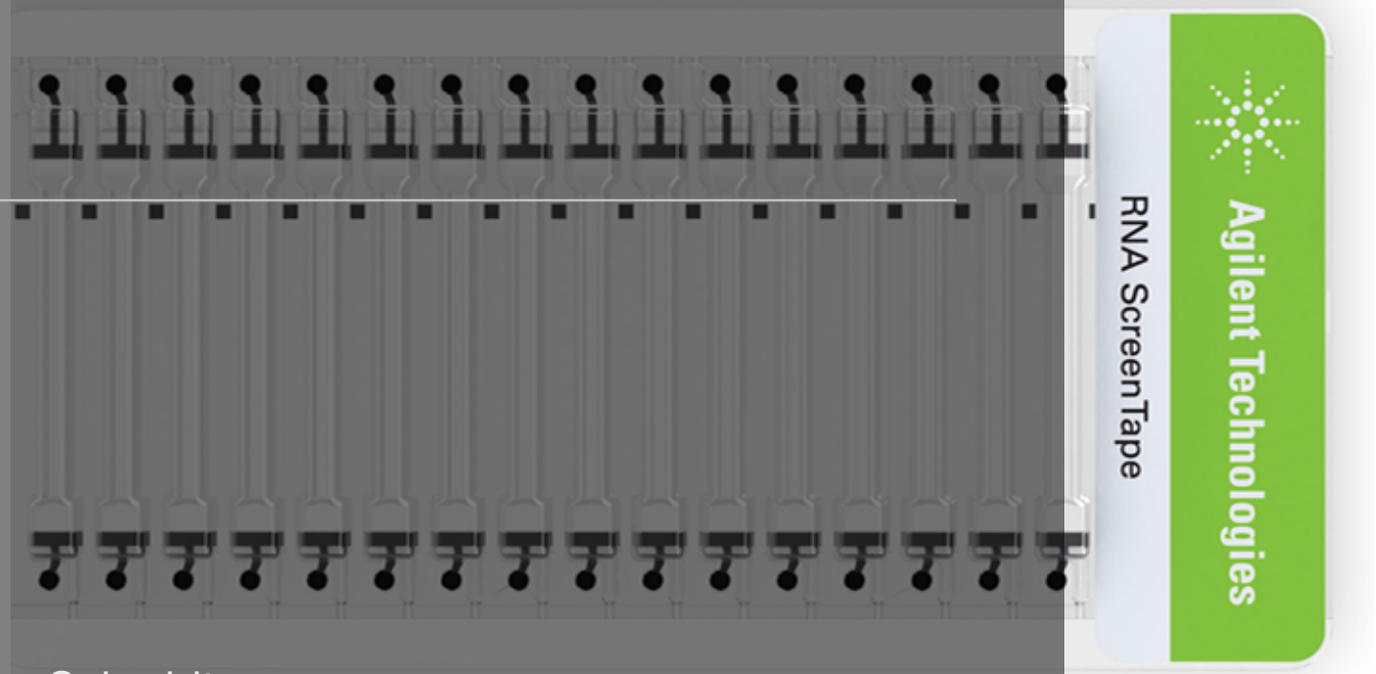
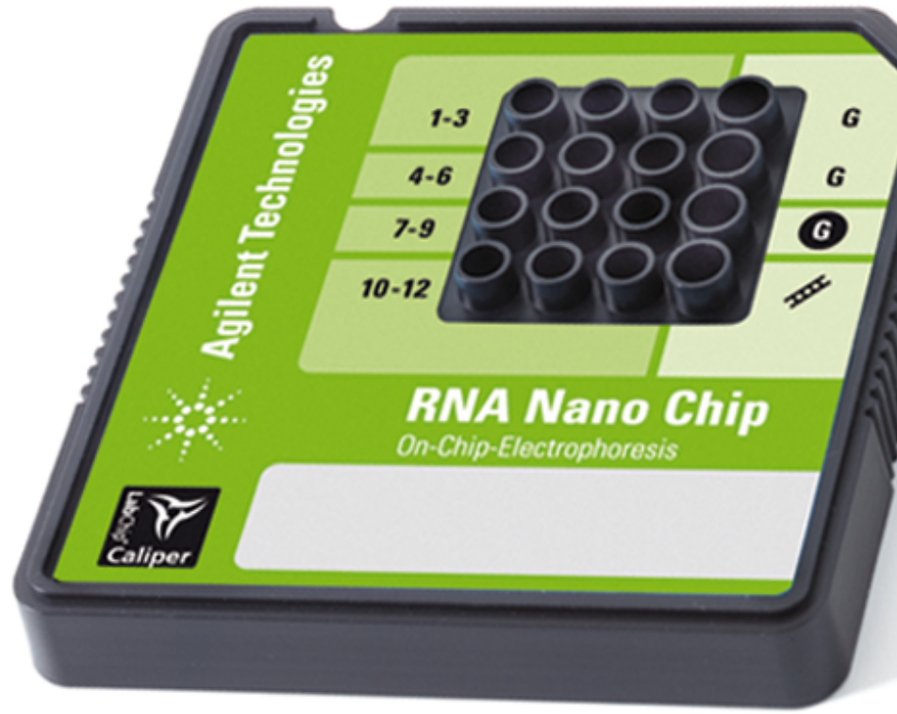
Chapter 3

RNA quality control





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Section 1

Introduction

INTRODUCTION

1. RNA concentration
2. RNA purity
3. RNA integrity
4. MIQE guidelines

Gene expression methods, such as quantitative real-time PCR (RT-qPCR), DNA microarrays and transcriptome sequencing, are techniques used to evaluate the activity of genes through determination of RNA transcript levels. Experiments are reliant on RNA extraction and subsequent analysis, with the resulting data reflecting a snapshot of gene expression at the moment of RNA extraction.

Meaningful gene expression analysis can only be obtained if the quality of the RNA sample is controlled. Important features contributing to RNA quality include:

- RNA concentration
- RNA purity
- RNA integrity

RNA concentration

For gene expression experiments, it is important to know the concentration of the starting material. Best practice dictates that equivalent sample amounts are used when comparing the results from different input samples. Furthermore, determining the concentration of the starting material allows for optimization of subsequent experimental steps.

RNA purity

Samples can be contaminated with proteins, genomic DNA or other organic compounds. This contamination may affect the ability to quantify the RNA. The extent of interference from the contamination will depend on the selected quantification method. Certain extraction kit buffers can have a negative effect on RNA quantification of high sensitivity assays due to high salt concentrations (Agilent Technologies, 2004). In addition, downstream applications and the relevance of their results may also be affected by such contamination. Contaminants in samples - genomic DNA, DNA binding proteins, phenolic compounds, or exogenous particles introduced during RNA extraction, e.g. powder from gloves - have been shown to inhibit downstream processing steps, such as reverse transcription and PCR amplification (Wilson, 1997).

RNA integrity

In contrast to DNA, RNA is a relatively unstable molecule. Therefore, RNA integrity is a crucial part of the overall quality of the RNA sample. The instability of the RNA sample is a consequence of the basic structure of RNA molecules. Like DNA, RNA is a polymer chain that is assembled from nucleotides. However, in contrast to DNA, the polymer backbone is composed of ribose rather than deoxyribose. Ribose carries a hydroxyl group attached to the pentose ring in the 2' position. These hydroxyl groups are more prone to non-enzymatic, alkaline hydrolysis, even in aqueous solutions at neutral pH. The 2'-hydroxyl group can react with the adjacent phosphodiester bond to cleave the backbone of the RNA molecule. To avoid hydrolysis, RNA samples have to be carefully stored, either at very low temperatures, precipitated or most preferably, freeze-dried.

Breakdown of RNA can additionally be catalyzed by ribonuclease (RNase). These enzymes are often very stable and widely distributed. RNases are present in cells and regulate the cellular RNA metabolism. Such endogenous RNases must be immediately inactivated upon tissue harvesting and cell lysis to prevent RNA degradation. Other RNases are found in the environment, such as from secretions by the human skin.

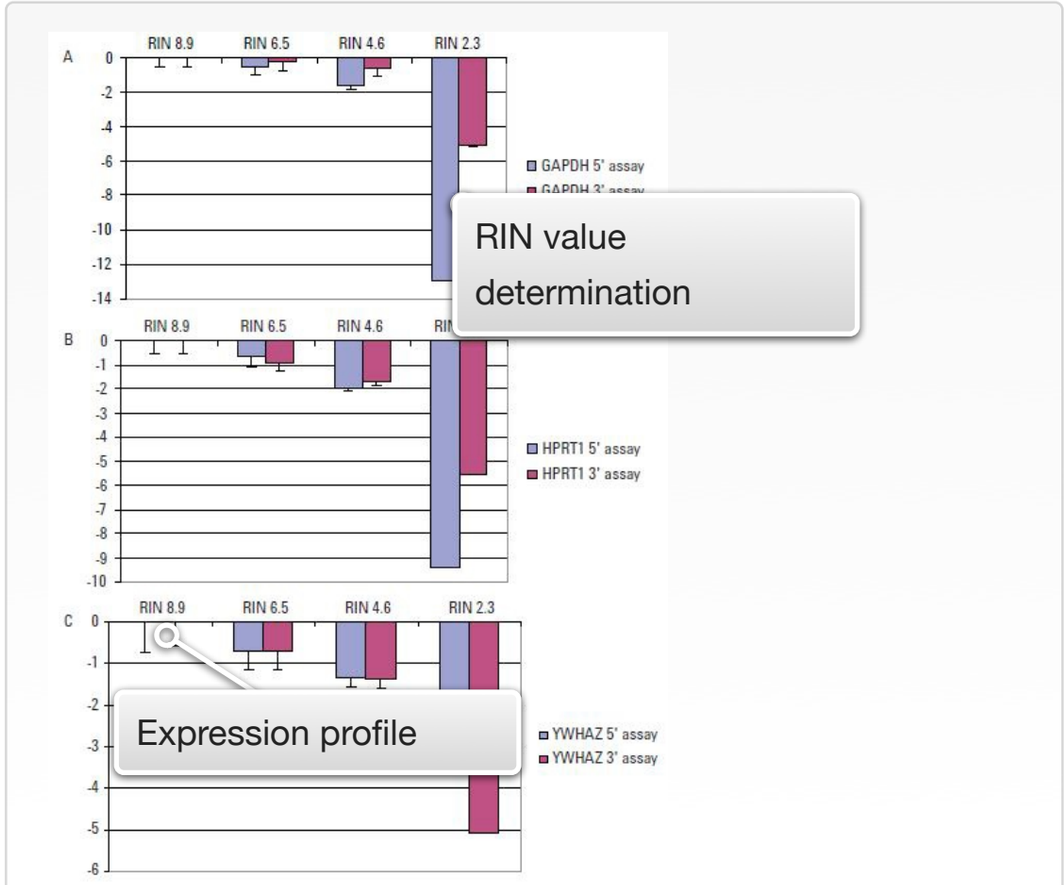
Therefore, it is mandatory to reduce the exposure to environmental RNases through the usage of gloves and RNase-free material.

RNA degradation not only reduces the amount of RNA within the sample, but more importantly degradation is sequence specific. Slightly degraded RNA may bias gene expression results. This bias may result from RNases targeting single-stranded RNA because most RNAs contain self-complementary sequences that allow parts of the RNA to fold and hybridize with itself to form intra-strand double helices, which are partially stable and less prone to enzymatic degradation. In addition, RNases differ in their substrate specificity for certain nucleotides or sequences. Several studies have clearly shown that the initial integrity of the starting RNA material significantly affects the results of downstream applications, see **Interactive 3.1** (Auer et al., 2003; Imbeaud et al., 2005; Fleige et al., 2006; Müller, 2008).

In addition to optimal RNA quality, it is critical to ensure that each stage of the experimental process is optimized to produce reliable RT-qPCR data. All prior and subsequent steps require validation, including primer design, PCR efficiency testing, linear dynamic range, limit of detection, and precision are all important considerations for a successful RT-qPCR experiment.

Interactive 3.1 The integrity of the RNA templates influences the outcome of the RT-qPCR experiments. Blue: 5' assay; Violet: 3' assay. A) GAPDH, B) HPRT1, C) YWHAZ) The Y-axis scale is in log2.

Müller S. Optimizing real-time quantitative PCR experiments with the Agilent 2100 bioanalyzer. Agilent Technologies Application Note 2008: 5989-7730EN



MIQE guidelines

Original publication: MIQE guidelines

In order to compare gene expression data, it is essential to use reliable standardized methods, including methods for quality control. The ultimate goal is to establish recording and reporting standards for gene expression data. One example of this effort is the 'Minimum Information About a Microarray Experiment' (MIAME) (Brazma et al., 2001). These guidelines describe the minimum information required to ensure microarray data can be easily interpreted and results can be independently verified. Similarly, the MIQE (Minimum Information for publication of Quantitative real-time PCR Experiments) guidelines are setting standards for RT-qPCR by describing the minimum information required to objectively evaluate robustness of a given RT-qPCR experiment. Both approaches emphasize the strong need for reliable RNA quality control, as the quality of the extracted RNA as starting material is a major concern for successful gene expression studies. MIQE stresses that the experimental data presented should also include information about instrumentation, methodology of RNA extraction and the subsequent quantification, purity and integrity of the RNA sample, as well as other essential information about experimental conditions and assay characteristics. This increased level of experimental information and transparency not only helps in producing more reliable gene expression data, but also allows standardization of experiments between different laboratories ensuring that data can be reproduced consistently.

Precautions to ensure high quality RNA samples are described above, but since sampling techniques, sample storage and treatment, as well as RNA extraction procedures can vary significantly, it is essential that a reliable method is used for the analysis of starting material. The MIQE guidelines recommend determining the quality of extracted total RNA to ensure reliable and consistent data from RT-qPCR experiments.

Recommendations to ensure high quality RNA

• *Avoid RNAse contamination*

– *Wear gloves at all times and change frequently*

– *Use RNAse free tubes, tips, water, and reagents*

– *Use an "RNase-free location" with dedicated equipment for RNA extraction*

– *Separate pre-PCR and post-PCR location*

• *Decrease tissue sampling time to a minimum*

– *Rapidly freeze samples after harvest in liquid nitrogen*

– *Add disrupting lysis buffer*

– *Add RNA Stabilization Solution*

• *RNA extraction*

– *Make sure tissue is frozen during lysis*

– *Use RNAse inhibitors*

• *Use appropriate storage conditions*

– *Stable for 1 year in aqueous solution at -20°C*

– *Long-term storage as salt/alcohol precipitate at -80°C*

– *Consider RNA inhibitors for storage*

– *Avoid freeze-thaw cycles*

Section 2

Methods

METHODS FOR RNA QUALITY CONTROL

1. Overview of RNA quality control methods
2. Spectrophotometer
3. Fluorescent dye based assays
4. 3´:5´ integrity assay
5. Agarose Gel Electrophoresis
6. Capillary Electrophoresis
7. Numerical assessment of the integrity of RNA

RNA quality control can be performed using widely different approaches, which differ significantly in their suitability for this task. For example, it has been demonstrated that results vary significantly if the same samples are quantified using different methods. This is because certain approaches cannot discriminate RNA from other sample contaminants, such as genomic DNA and phenol (Lightfood, 2002). Therefore, it is essential to choose one specific protocol for RNA quality control and use this approach for all samples within a given experiment.

Overview of RNA quality control methods

	Spectro-photometer	Fluorescent dye detection	Agarose Gel Electrophoresis	Capillary Electrophoresis	3´:5´ integrity assay
Concentration	X	**X**		X	
Purity	X [1]		X [2]	X [2]	
Integrity			X	**X**	**X**

X suggested method according to the MIQE guidelines

[1] Determination of impurities including proteins, DNA or organics

[2] Detection of contaminating DNA

Spectrophotometer

Total RNA can be quantified using a spectrophotometer by taking advantage of the strong adsorption of UV light by nucleic acids. Unfortunately, this UV-spectroscopic method does not discriminate between different types of nucleic acids, therefore contaminants – most commonly, genomic DNA - can significantly skew any results. For more precise RNA quantification, contaminating DNA must be removed by a DNase. Free nucleic acids can also contribute to increased absorption at 260nm, so further purification of the total RNA after DNase treatment may be required.

In addition to quantification, the purity of a total RNA sample can be determined by measuring the absorbance at 260 and 280 nm (A260/280), as well as 230 and 260 nm (A230/260). The ratio of absorbance at 260 and 280 nm can indicate protein or phenol contamination. Pure total RNA has an A260/280 ratio of 2, but ratios greater than 1.8 are usually considered an acceptable indicator of RNA purity (Sambrook et al., 1989; Manchester, 1996). The absorbance of RNA at 260 nm is not dependent on the pH of the solution. However, some typical contaminants, such as protein or phenol, have an absorbance at 280 nm that is pH-dependent. This means that the A260/A280 ratio will vary depending on the pH of the solution. Besides pH, the A260/A280 ratio is also susceptible to the ionic strength of the sample solution. Therefore, it is important to only compare results from samples measured in the same buffer and to use exactly the same buffer to dilute the samples and use as the blank. MIQE guidelines recommend measuring the A260/280 ratio in a buffer at neutral pH.

Less frequently, the ratio of absorbances at 230 and 260 nm is used to indicate residual contamination by organic compounds. Typical sources of contaminations that adsorb at 230 nm are proteins, chaotropic salts, like guanidinium isothiocyanate, and phenol. These contaminations are either already present in the tissue sample or are introduced through the lysis solution.

UV spectroscopy is the most widely used method to quantitate total RNA due to its simplicity, and with UV spectrophotometers available in most laboratories. However, this method has several drawbacks, as described above. In addition, conventional spectrophotometers use cuvettes which often require a significant amount of potentially precious RNA sample for analysis. To address this need, spectrophotometers have been developed which require very low sample volumes, typically 1 to 2 µL. Another weakness is the limited sensitivity range of UV spectroscopy, which may result in unreliable measurements of experimentally relevant RNA samples of low abundance.

In summary, UV spectroscopy may be suitable for the determination of total RNA quantity and purity; however, this method cannot provide any information on RNA integrity.

Fluorescent dye based assays

An alternative and highly sensitive method to assess RNA concentration is to measure the fluorescence intensity of dyes that bind to RNA and selectively fluoresce when bound, for example RiboGreen® (Jones et al., 1998). The stained samples can be quantified with a fluorimeter, either in cuvettes or microplates. This method may also be combined with a quantitative PCR system that has an integrated sample fluorescence detection function.

Contaminating genomic DNA can affect the results, so for more exact RNA quantification the removal of contaminating DNA with DNase treatment is necessary. Furthermore, fluorescent-dye-based total RNA quantification only measures polymeric nucleic acids and will not detect contaminating proteins and free ribonucleotides. This sensitive method is useful for samples with very low RNA abundance. It also allows for more automated and higher throughput measurements. Fluorescence based assays are recommended by the MIQE guidelines for RNA quantitation. However, it is only suitable for total RNA quantification, and will not provide information on RNA purity and integrity.

3´:5´ integrity assay

A 3´:5´ assay using GAPDH as the target sequence has been proposed as an alternative measure of mRNA integrity (Nolan et al., 2006). GAPDH mRNA is converted to oligo-dT primed cDNA in three individual qPCR assays with target amplicons for the 5´ end, middle and 3´end regions of the GAPDH mRNA sequence. The described triplex assay is performed using TaqMan® chemistry with each amplicon detected by a target-specific, differentially labelled probe. The “3’:5’ ratio” describes the comparison of the signal intensity from the 3' probe over the 5' probe. This value reflects the integrity of the mRNA template as the generation of cDNA by the reverse transcriptase would be prematurely terminated if the mRNA is degraded. A 3´:5´ ratio of around 1 indicates high mRNA integrity, whereas a value greater than 5 suggests mRNA degradation.

The 3´:5´ assay measures the integrity of the GAPDH mRNA as a representative of the integrity of all mRNAs present in an RNA sample. The data obtained is independent of ribosomal RNA integrity and provides a quantifiable measure of mRNA degradation. This assay is particularly applicable for analysis of precious samples when little RNA is available. However, since different mRNAs degrade at different rates, the degradation of one reference mRNA may not always reflect the overall mRNA integrity.

Agarose Gel Electrophoresis

Agarose gel electrophoresis is widely used for qualitative analysis of nucleic acid fragments. It is a gel-based technique offering a broad separation range of nucleic acid molecules based on size and charge. During RNA electrophoresis, negatively-charged RNA migrates through the gel towards the anode. Simplistically, the length of a RNA molecule determines its migration time. However, secondary structure formation of RNA, via intra-molecular base pairing, may delay migration, therefore, it is recommended to perform RNA electrophoresis under denaturing conditions.

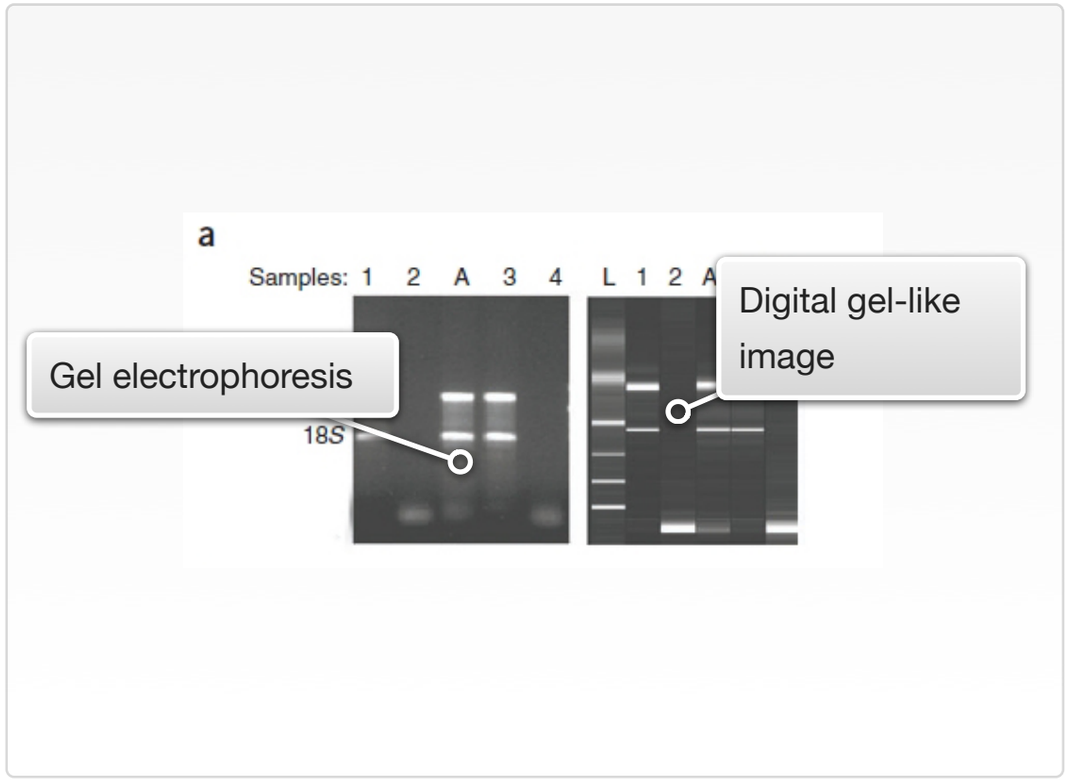
For detection, RNA within the gel is stained with ethidium bromide or a highly sensitive fluorescent dye (e.g. SYBR® Green II or SYBR® Gold). These intercalating dyes fluoresce when exposed to light. Ethidium bromide is a potential mutagen, carcinogen, and teratogen requiring safety precautions and disposals hazardous waste. SYBR®-based dyes are more sensitive and less harmful alternatives to ethidium bromide. The stained gels are viewed with a transilluminator. A transilluminator system may also include image capture devices, such as a digital or Polaroid camera, that allow an image of the gel to be taken or printed for further analysis using specific gel analysis software.

Total RNA separated on an agarose gel demonstrates characteristic species-specific patterns of bands (**Interactive 3.2**). Typically, in eukaryotes, two major bands comprising the 28S and 18S ribosomal RNA (rRNA) are detected. Smaller RNA species can be observed, depending on the extraction method. Total RNA was considered of high quality when the 28S: 18S rRNA ratio equalled 2 or higher (Sambrook et al., 1989). However, this approach relied on manual interpretation of gel images, a subjective method of calculation suspect to human error and made comparing samples from one lab to another a challenge. Furthermore, many studies found ribosomal ratios showed only weak correlation with RNA integrity (Imbeaud et al., 2005; Miller et al., 2004).

Agarose gel electrophoresis is relatively inexpensive, easily accessible and provides a visual inspection of the total RNA. However, the MIQE guidelines do not recommend the use of agarose gel electrophoresis due to the lack of standardization and objectivity. In addition, agarose gel electrophoresis requires significant amounts of precious RNA sample, involves the handling of hazardous chemicals, and does not provide RNA quantification.

Interactive 3.2 Typical presentation of RNA separation by agarose gel electrophoresis (left) and the Agilent 2100 Bioanalyzer system (right). 500 ng per sample was loaded for all samples.

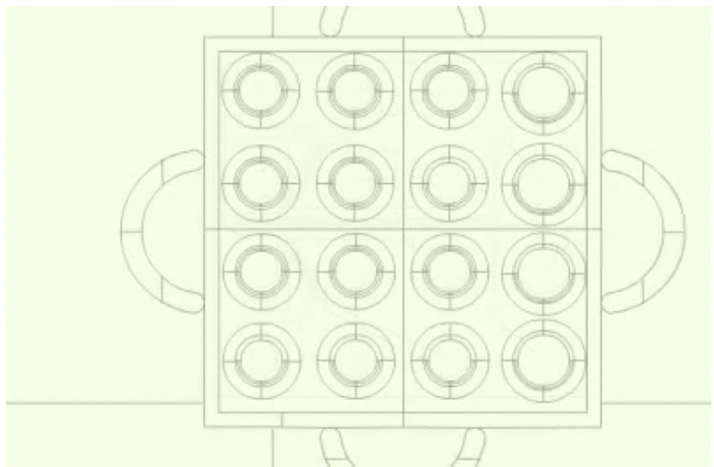
Masotti A., Preckel T. Analysis of small RNAs with the Agilent 2100 Bioanalyzer. Nature Methods 2006.



Automated Electrophoresis

Microcapillary electrophoresis can be used to separate, size and quantify RNA samples (Müller et al., 2000). The Agilent 2100 Bioanalyzer system was the first microcapillary platform available for RNA quality control utilizing on-chip electrophoresis. In contrast to classical gel electrophoresis, this format significantly reduced separation time, as well as reagent and sample consumption. During chip preparation, an interconnected network of micro-channels is filled with sieving polymer and fluorescence dye. After RNA samples and ladder are loaded, an electric field is applied to the micro-channels with electrophoresis performed in an automated and reproducible manner (Movie 3.1).

Movie 3.1 Micro channels use for capillary electrophoresis

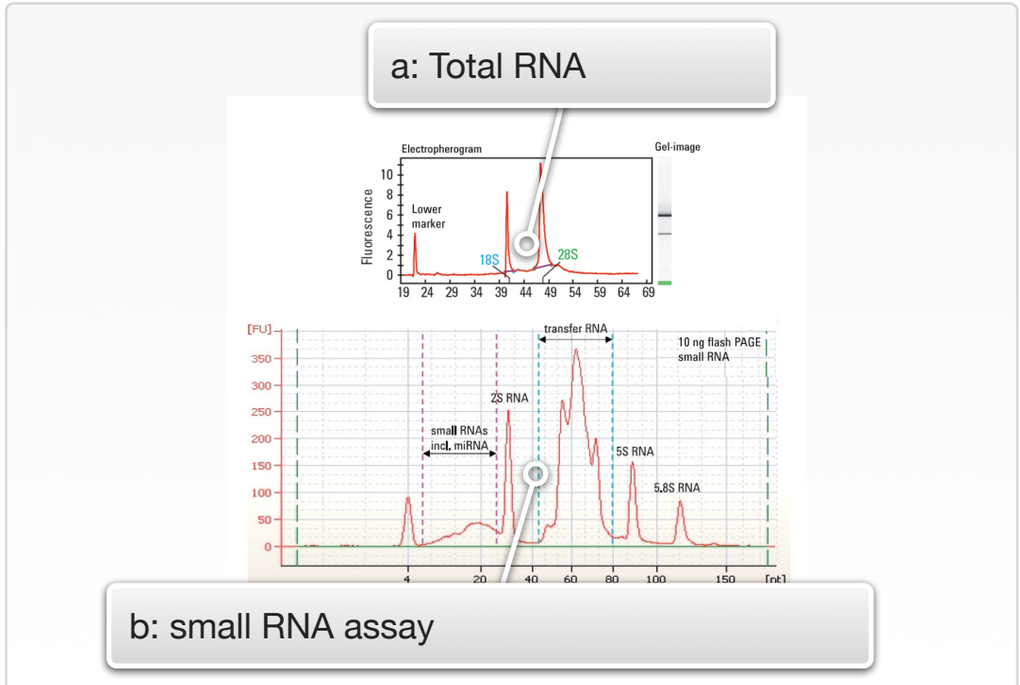


Interactive 3.3 The Agilent 2100 Bioanalyzer system provides RNA quality control results in both electrophoretic data as well as in gel-like images. Assays are available for the analysis of (a) total RNA and (b) small RNA and allows to separate various RNA species.

Agilent Technologies 2003. RNA LabChip kits. Fast quality control of RNA with minimal sample consumption. Agilent Technologies Technical Note. 2003: 5989-0229EN

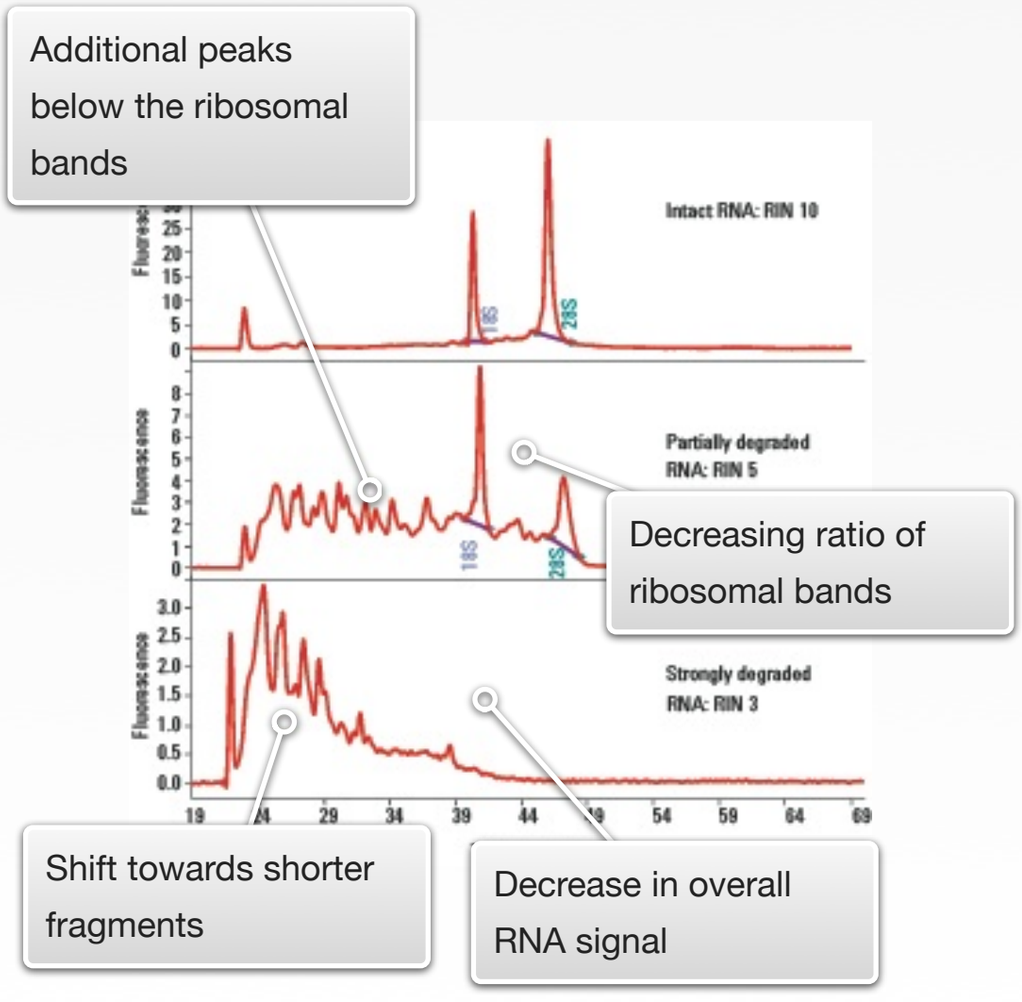
Sismeiro O, Coppée JY, Antoniewski C, Thomassin H. Analysis of small RNAs from Drosophila Schneider cells using the Small RNA assay on the Agilent 2100 bioanalyzer. Agilent Technologies Application Note. 2008: 5989-8539EN

Internal markers in the ladder and sample wells are used to align the data to compensate for possible drift effects. Various RNA species – such as individual ribosomal subunits (5S, 18S and 28S), tRNA, mRNA and fragmented RNAs – may be separated and visualized (Interactive 3.3).



Interactive 3.4 Electrophoretic traces of different degradation stages of RNA with the respective RNA integrity number (RIN).

Müller O, Lightfood S, Schröder A. RNA Integrity Number (RIN) – Standardization of RNA Quality Control. Agilent Technologies Application Note 2004: 5989-1165EN



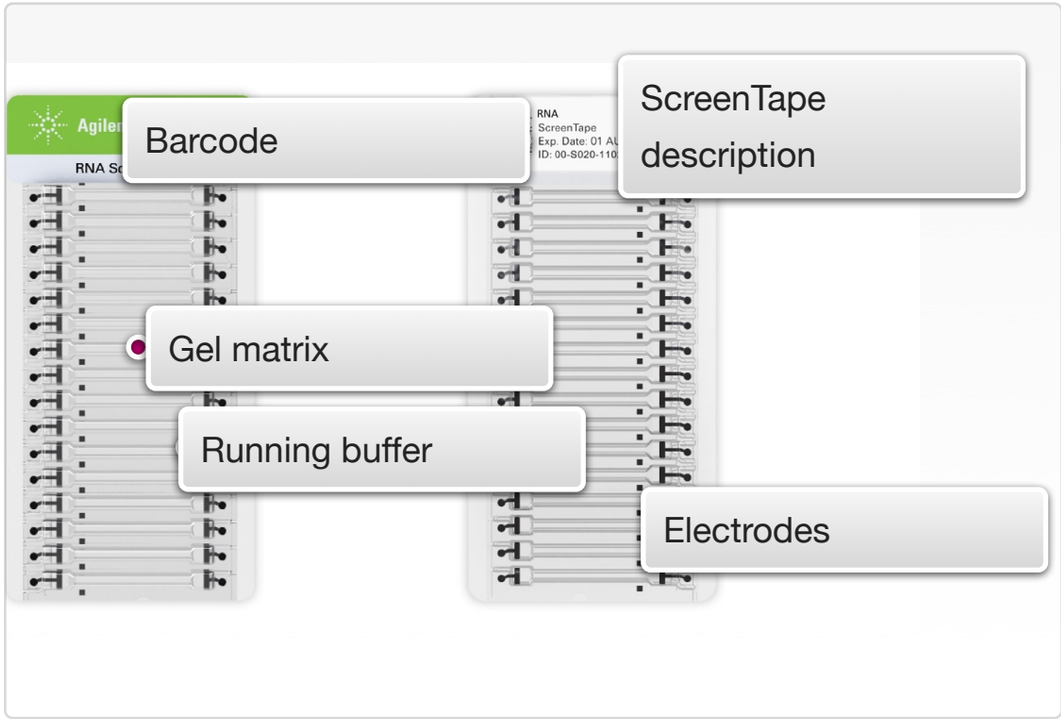
Electrophoretic traces reveal that progressing RNA degradation results in diminished signal intensities for the 28S and 18S ribosomal bands, in conjunction with an increase of shorter fragments, i.e. elevated baseline noise between the two ribosomal bands and below the 18S band (Interactive 3.4).

In addition to integrity assessment, the RNA assays for the Agilent 2100 Bioanalyzer system have an additional capacity to detect contaminating DNA, such as partially sheared genomic DNA (Abbaszade et al., 2003; Caruana et al., 2004).

The Agilent 2100 Bioanalyzer system has become widely adopted (see publication list), particularly for gene expression profiling platforms, as a convenient and objective way of assessing the quantity and integrity of RNA. As such, this approach is fully concordant with MIQE guidelines and often recommended for total RNA quality control.

In order to achieve higher sample throughput, by allowing for more automation with less hands-on preparation, other platforms use multi-parallel capillary systems for capillary electrophoresis. In addition, increased flexibility, in terms of sample-throughput scalability, can be offered by platforms which utilize prefilled multi-lane gels (**Interactive 3.5**). The Agilent 2200 TapeStation system and the respective ScreenTape assays provide an automated individual channel electrophoresis technology, facilitating variable sample throughput.

Interactive 3.5 The Agilent RNA ScreenTape assay is used in combination with the 2200 TapeStation system for the determination of quality and quantity of total RNA samples from eukaryote or prokaryote origin.



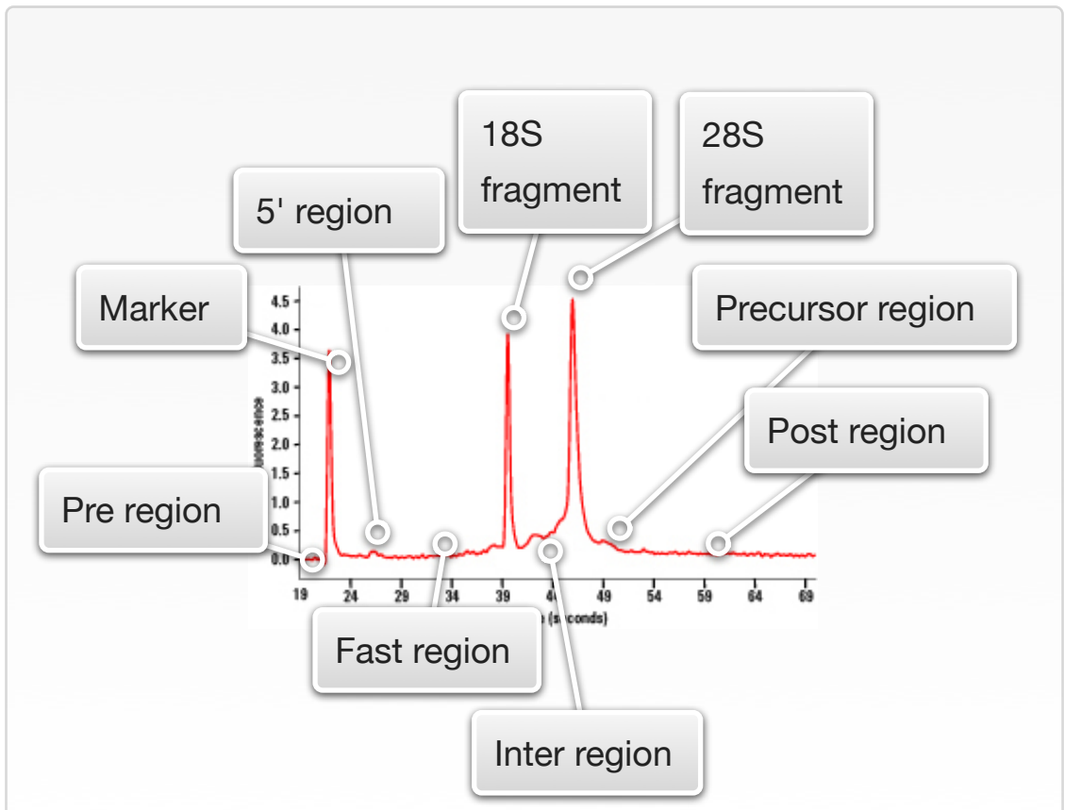
Thus, capillary electrophoresis can provide analysis of RNA integrity and concentration, as well as determination of DNA contamination in a single step, with various degrees of automation depending on the system chosen. In addition, capillary electrophoresis systems all provide digital data output, allowing for greater consistency and removing the subjective interpretation of results by individuals.

Numerical assessment of the integrity of RNA

By taking advantage of the digital data obtained with the Agilent 2100 Bioanalyzer system, an objective, numerical measure of RNA quality and integrity was developed for eukaryotic total RNA (Schröder et al., 2006). The 'RNA Integrity Number` (RIN) algorithm of the 2100 Expert software processes the digital data based on the acquired raw data and calculates RIN. The underlying algorithm ranks features extracted from electrophoretic traces according to their information (Interactive 3.6). The information content of 1300 eukaryotic RNA samples was used to train and select a prediction model on the basis of an adaptive learning tool (Müller et al., 2004). Using this tool, sample integrity is no longer determined by the ratio of the ribosomal bands, but by the entire electrophoretic trace of the RNA sample, including the fraction of the area in the region of 18S and 28S rRNA, the height of the 28S peak, the presence or absence of RNA degradation products, the fast area ratio and marker height. A RIN value of 10 indicates that the tested RNA sample is fully intact, and not degraded or fragmented. A RIN value of 1 represents a sample that is fully degraded. The assigned RIN is independent of sample concentration, instrument and analyst, thereby becoming a real standard for RNA integrity.

Interactive 3.6 The RNA Integrity Number (RIN) is calculated by a dedicated software algorithm to assess the quality of RNA preparations. This electrophoregram details the regions that are evaluated for RIN calculation. The algorithm was created out of a large collection of RNA data of various tissues and quality.

Müller O, Lightfood S, Schröder A. RNA Integrity Number (RIN) – Standardization of RNA Quality Control. Agilent Technologies Application Note 2004: 5989-1165EN



Specific RIN requirements and threshold levels for successful respective downstream application have been reported by various investigators for different organisms and specific sample types. Some examples include: Fleige et al., 2006 demonstrated that RT-qPCR performance is affected by RNA integrity, whereas PCR efficiency is generally not affected. For their downstream applications using mammalian tissues, a RIN greater than 5 is considered as good total RNA quality and a RIN greater than 8 as intact total RNA. Ribeiro-Silva et al., 2007 showed that RNA isolated from ten year old formalin-fixed paraffin-embedded (FFPE) tissues yielded RNA integrity values similar to extracts from months-old samples. These investigators found a minimum RIN value of 1.4 from FFPE samples was sufficient for successful execution of their downstream RT-qPCR tests. For bacterial RNA, Jahn et al., 2008 reported that RIN values below 7 resulted in high variation and loss of statistical significance when gene expression was analyzed by RT-qPCR. As evidenced by the variety of thresholds for different applications, it is critical for investigators to determine the RIN thresholds for their particular sample type and downstream experimental methods.

The RNA Integrity Database (RINdb) is a freely accessible repository holding hundreds of user submitted total RNA traces. The RINdb allows viewing "normal" total RNA profiles for different tissue types, and comparing the effects of using different RNA extraction methods and kits.

An alternative to RIN is the RIN equivalent (RINe) obtained with the Agilent 2200 TapeStation system. RINe provides an accurate and objective assessment of total RNA degradation, giving comparable values to RIN (Wilkes et al., 2010; Agilent Technologies, 2011; Padmanaban 2012; Connelly et al., 2013).

Meanwhile, similar to RIN and RINe, other numerical assessments of the integrity of RNA have been developed, emphasizing the importance and increased interest for standardization (Vermeulen et al., 2011) as highlighted by the MIQE guidelines.

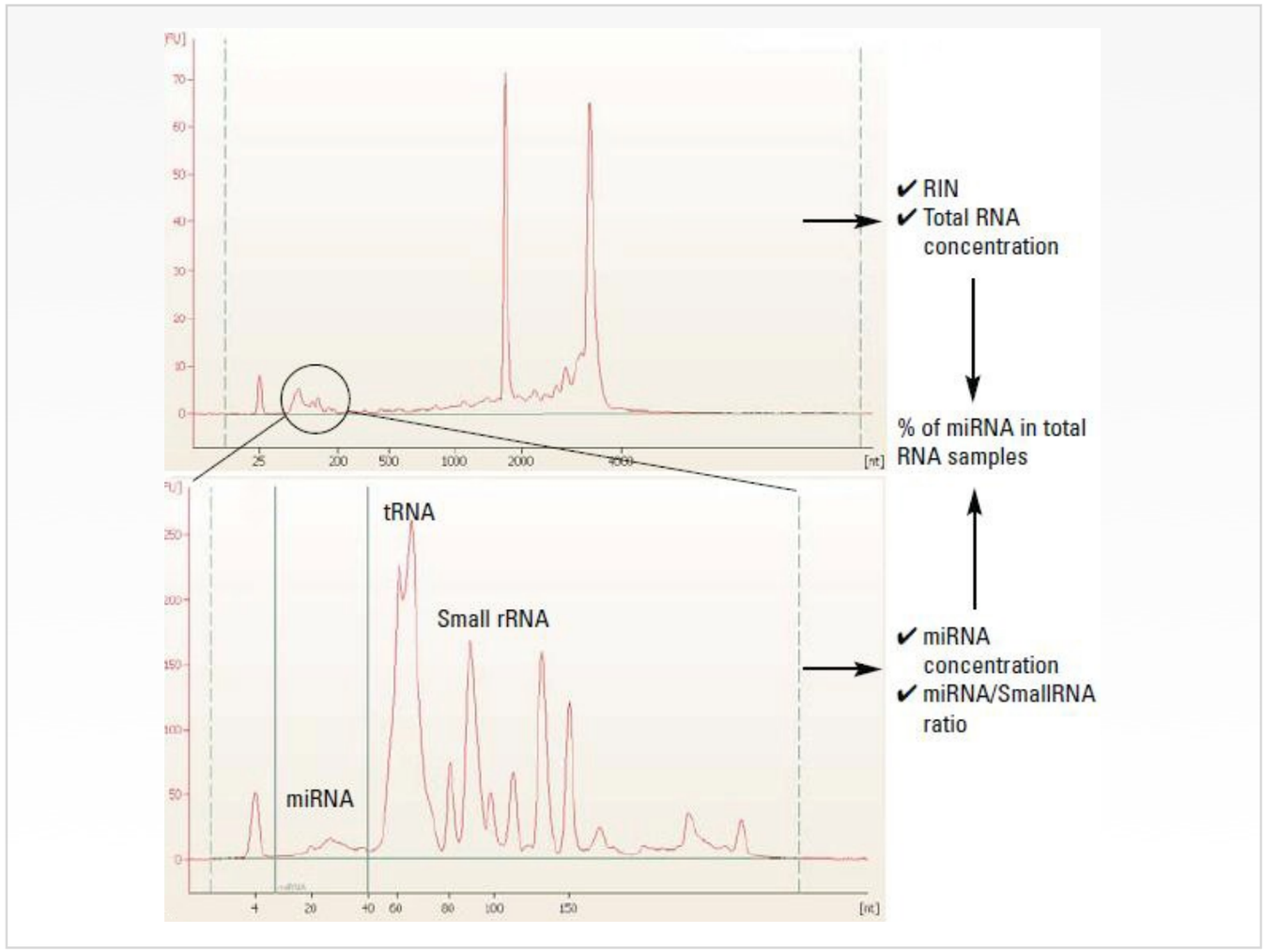
Section 3

Quality control of miRNA

The determination of RNA quality should also be routinely applied to other applications, such as microRNA (miRNA) expression profiling. Conventional photometric methods are not suitable to determine the concentration of these short, non-coding regulatory RNA molecules. The Agilent 2100 Bioanalyzer system allows for the selective determination of the concentration of miRNA and small RNAs up to 150 nucleotides (Interactive 3.7) (Becker et al., 2010; Tissot, 2008). It is important to understand that RNA extraction methods retain miRNA and small RNA fractions. As miRNA and small RNA migrate similar to degraded total RNA high concentration of these species may thus yield lower-than-expected RIN values (Becker et al., 2010). Determination of RIN thresholds may need to be adjusted for specific extraction methods. Ibberson et al., 2009 reported to use totalRNA samples with a RIN equal to or above 7 for the specific profiling of miRNAs.

Interactive 3.7 miRNA analysis workflow. All RNA quantities (top: RNA 6000 Assay, bottom : Small RNA assay) were analyzed to estimate the % miRNA in total RNA samples.

Tissot C. Analysis of miRNA content in total RNA preparations using the Agilent 2100 bioanalyzer. Agilent Technologies Application Note. 2008: 5989-7870EN



Review - RNA quality control

Question 1 sur 4

What RNA quality characteristics can be checked using a spectrophotometer

- ✅ **A.** Purity
- ✅ **B.** Concentration
- ⚪ **C.** Integrity
- ⚪ **D.** GC content

◀ Répondre ▶

Section 4

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Chapter 4

Reverse Transcription

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Section 1

Introduction

History and general description of Reverse Transcriptases

Reverse transcription is the copying of an RNA template into a complementary DNA, or cDNA, by an RNA-directed DNA polymerase. Reverse transcriptases, or RTs, were initially discovered in RNA tumor viruses in independent investigations by Howard Temin at the University of Wisconsin and David Baltimore at the Massachusetts Institute of Technology (Temin and Mizutani, 1970; Baltimore, 1070). The concept of a DNA polymerase capable of recognizing RNA templates was initially controversial as it upended the “central dogma” of the day that molecular biological information flowed in one direction only: DNA to RNA to protein. In the years since, the central role of reverse transcription in RNA biology has become universally recognized and reverse transcriptase (RT) is an indispensable component in the enzymatic “tool box” used by virtually all researchers studying RNA.

In addition to its RNA-dependent DNA polymerase activity, which is primer-dependent and requires a magnesium or manganese cofactor, RT enzymes have other features of importance to researchers. These include DNA-dependent DNA polymerase activity and RNase H activity which degrades the RNA component of RNA:DNA hybrids. Unlike typical DNA polymerases, RTs lack a proofreading function. Their presence in PCR experiments can inhibit Taq polymerases functionality in the reaction, leading to inaccurate results.

Types of Reverse Transcriptases

The most commonly used commercially available RTs come from avian myoblastosis virus (AMV) and Maloney Murine leukemia virus (M-MLV).

AMV RT is composed of two subunits and requires six 10 mM divalent cations for optimal activity. Relative to other types of RT, it is more processive and has a higher optimal activity temperature range (42 to 48°C), making it more suitable for reverse transcribing RNAs with strong secondary structure. However, AMV RT has relatively high RNase H activity, making it less useful for synthesizing long transcripts. In contrast M-MLV RT is composed of a single subunit and has lower RNase H activity. Because of these properties, M-MLV RT is frequently used for longer transcripts. M-MLV RT has an optimal activity temperature of 37°C. A number of M-MLV variants are available, including RNase H- point mutants that are more thermostable and ideal for difficult templates.

'Choosing the Right Reverse Transcriptase'

Another commercially available RT-like enzyme is Tth polymerase, a thermostable enzyme that functions as an RNA-dependent DNA polymerase in the presence of manganese, but as a DNA-dependent DNA polymerase in the presence of magnesium.

Applications and uses

Workflows

Reverse transcription can be used to create cDNA suitable for a variety of downstream applications.

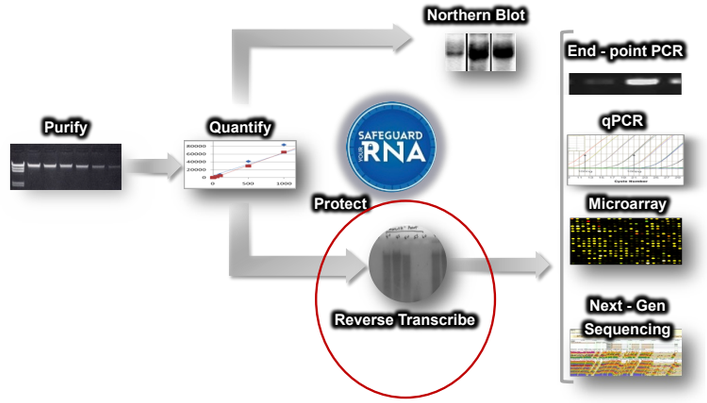


Figure 1: Reverse Transcription & RNA Workflow: The success of the RT reaction depends on the careful execution of multiple workflow steps each of which will affect final experimental results. The most reliable experimental results require high-quality RNA and reagents in experiments that are carefully planned and executed. Strict adherence to the MIQE guidelines will ensure that all critical experimental parameters are captured such that results may be properly interpreted.

RNA workflows (Figure 1) invariably require that RNA be isolated, or separated, from a sample type or matrix. Typically, the quantity and/or quality of the RNA sample is assessed prior to the setup of downstream analysis. We focus here on workflows where RNA is reverse transcribed to cDNA for further downstream analysis or processing. cDNA may feed into any number of downstream processing or analysis steps including end-point or real-time PCR, expression microarray analysis, next-generation sequencing (RNA-seq) or cloning. In some cases, reverse transcription and real-time PCR will be combined in a single reaction mixture which is referred to as one-step RT-qPCR. When reverse transcription and real-time PCR occur in separate reactions, the protocol is known as two-step RT-qPCR.

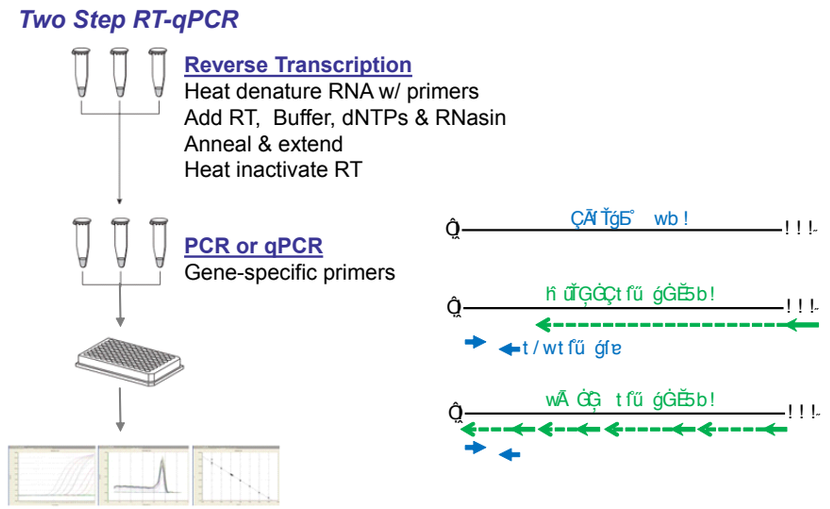


Figure 2: Reverse Transcription two-step RT-qPCR—Two-step RT–qPCR starts with heat denaturing the RNA template in the presence of primers. Reverse transcriptase, buffers, dNTPs and RNase inhibitors are added to the reaction, which is incubated at 25°C to promote annealing of primers to the RNA template and then heated to a temperature suitable for the RT enzyme to synthesize cDNA.

Two-step RT–qPCR starts with heat denaturing the RNA template in the presence of primers. Reverse transcriptase buffers, dNTPs and RNase inhibitors are added to the reaction, which is then incubated at 25°C to allow primers to anneal to the RNA template. Finally, the reaction is incubated at 37o to 42o, depending on the RT enzyme used, to facilitate cDNA synthesis. Priming of reverse transcription in two-step RT-qPCR workflows is typically performed with either oligo(dT) or random primers. Oligo(dT) will prime synthesis of cDNA from all polyadenylated messenger RNA molecules in an RNA pool. However, since this priming starts at the 3’end of the transcript, it is possible that amplicon sequences closer to the 5’ end of the transcript will be under-represented in the cDNA pool, because not all cDNAs will be full length. In contrast, use of random primers, typically hexamers, can theoretically facilitate synthesis of cDNAs representing all components in an RNA pool. This approach is less sensitive to RNA integrity or transcript length concerns. Following reverse transcription cDNA is then used as input for PCR or quantitative PCR (qPCR) applications.

This two-step process is preferred when a pool of cDNA is desired for the study of multiple transcripts via PCR or qPCR.

In the one step RT-qPCR approach, reverse transcription and qPCR amplification of target sequences are performed in the same reaction well or vessel. Instead of random hexamer or oligo(dT) primers target-specific primers are used for both reverse transcription and PCR.

One -step RT-qPCR requires less sample relative to the two-step method. Further, variance among any technical replicates can be used to assess the combined variability of both enzymatic steps. For these and other reasons one- step RT- qPCR is frequently used for quantification and quality control of RNA. A possible drawback to the one-step approach is that primer design may be more complicated because primers must function both at reverse transcription and PCR temperatures.

Considerations

RT-PCR First–strand cDNA synthesis

Protocol for Reverse Transcription

Reverse transcription is the starting point for other workflows such as creation of cDNA libraries, microarray analysis or RNA-seq. For RNA-seq applications, novel cDNA synthesis strategies may be employed, depending on which RNA population is the target of study.

Ideally, every RNA or mRNA molecule targeted for transcription will be converted into one cDNA molecule or, at the least, all target RNAs will be transcribed with the same efficiency. However, variability in RT efficiency, whether due to RNA sample quality, primer design, choice of RT enzyme, or other factors, can affect the degree to which the population cDNAs produced represents the distribution of RNA molecules in the sample. Variation in RT efficiency is a greatly under-appreciated factor in overall results quality (Bustin et al., 2015) with implications extending to large numbers of published gene expression studies. This reinforces the necessity of being rigorous in the recording and reporting of key experimental parameters, such as sample, nucleic acid extraction, and reverse transcription details, as outlined in the MIQE guidelines so that the validity RT-qPCR results can be properly interpreted.

Parameters for Successful Reverse Transcription

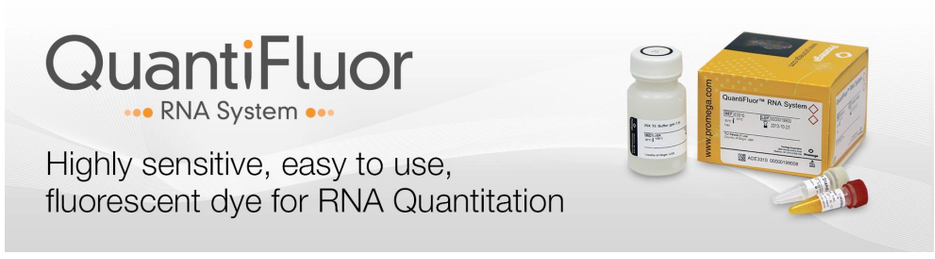
RNA quality has a profound impact on downstream results (Vermeulen, et al., 2011) and factors influencing sample quality are critical for the overall success of reverse transcription-based workflows.

RNA Purification and Purity

There are a number of parameters to consider when choosing an RNA extraction method, but ideally RNA samples should be free of contaminating proteins, such as nucleases, that may interfere with cDNA synthesis and downstream applications. Yield may be a primary consideration, however efficient removal of genomic DNA (gDNA) may be equally important. A purification method which generates large quantities of RNA but is inefficient at removing gDNA may not offer a real advantage over a method with lower yield but higher purity. This is particularly true in RT-qPCR applications, given that qPCR detects molecules across a 106 fold or greater range. Even low amounts of gDNA contamination can cause variability in qPCR results, while high amounts of gDNA contamination can cause outright quantitation errors.

One way to reduce concerns about gDNA contamination is to include a DNase step in the RNA isolation method. A DNase treatment step can be added at the end of any method. In addition, a no-reverse transcriptase control (no RT) should be used to determine whether gDNA is present in the RNA sample. Amplification products from “no RT” controls are due to the presence of gDNA, and indicatethat a similar quantity of gDNA is also present in the experimental samples. Genomic DNA contamination can lead to false positives in RT-PCR analysis. Removing gDNA by DNase treatment during or immediately after RNA isolation can improve PCR results.

RNA purity and quantity are typically assessed by absorbance measurements. Absorbance at 260nm (A260) is used to determine RNA quantity, and the ratio of absorbance at 260nm and 280 nm (A260/A280) is used as a measure of purity. High purity RNA should have an A260/280 ratio >1.8. Spiking experiments, in which RNA of unknown purity is added to an RT-qPCR that has amplified consistently in the past, can be used to detect inhibitors. Finally, quantifying RNA will help normalize the quantity of RNA you are adding to each reaction. Specialized instrumentation, such as the Promega Quantus® used with the QuantiFluor® RNA System, can streamline the absorbance-based determination of RNA concentration in samples.



RNA integrity

The intactness, or integrity, of an RNA sample is critical for many downstream applications. An important feature of intact eukaryotic RNA is the presence of both 28S and 18S ribosomal RNA (rRNA) subunits (Figure 3). The presence of these bands and overall RNA quality may be assessed by agarose gel electrophoresis and other methods.

Ideally, the 28S band should be nearly twice as bright as the 18S band when visualized on an agarose gel although roughly equal brightness indicates RNA of sufficiently high quality for most applications. Other parameters indicative of RNA quality that can be evaluated using agarose gel electrophoresis include the presence of a high molecular weight band, which can indicate contaminating gDNA, the presence of a low molecular weight smear, which is a sign of RNA degradation, and the visible smearing of the rRNA bands, which also indicates RNA degradation.

A more objective way of determining RNA quality is to use the Agilent BioAnalzyer, a microfluidics-based system which performs an electrophoretic analysis of samples to produce an electropherogram that reveals the relative abundance of RNA components, such as the 28S and 18S RNA, and degradation products.

Bioanalyzer Application ‘RNA Integrity Number (RIN) – Standardization of RNA Quality Control

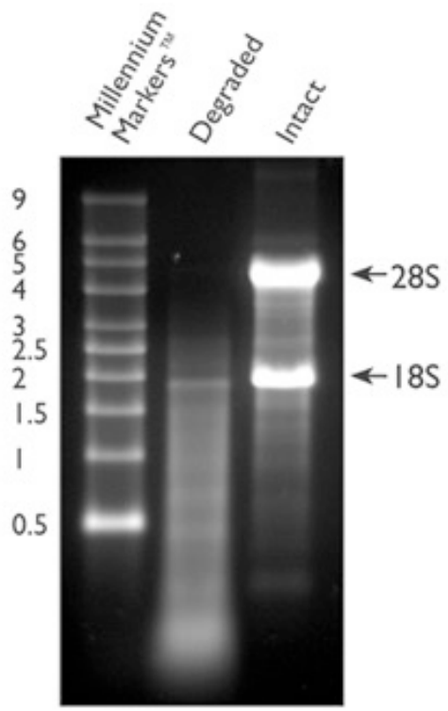


Figure 3: RNA integrity: Electrophoresis of denatured total RNA stained with SYBR green dye can be used to assess quality and quantity of RNA based on relative intensity of 28S and 18S rRNA bands. Degraded RNA results in a low molecular weight smear

More information on Methods for RNA Quality Assesment

The instrument software uses these relative quantities to calculate an RNA Integrity Number (RIN) which may vary from 1 to 10. RIN scores of 8 and above indicate RNA of high quality suitable for most applications. Lower RIN values are indicative of RNA that is degraded and may or may not be suitable for analysis, depending on experiment. For instance, detection of rare messages may be problematic with RNA with a RIN =7 or lower.

RNA degradation concerns can be minimized by following procedures for creating and maintaining a ribonuclease-free (RNase-free) environment (Blumberg, 1987). Use of an RNase

inhibitor (e.g., Recombinant RNasin® Ribonuclease Inhibitor) is strongly recommended.

Don't Let RNases Ruin Your Week(end): Establish a Ribonuclease-Free Environment

RT Primers

cDNA synthesis typically involves use of one of three types of primers: Oligo(dT), Random Hexamers or Gene-Specific Primers Oligo(dT) primers anneal specifically to the poly(A) portion (3´-end) of mRNA molecules.Random Hexamers, which are short oligonucleotides of random sequence, may be preferred for transcripts that are long (>4kb) or lack a poly(A) tail such as the case with prokaryotic mRNA. These can prime reverse transcription at multiple points along the transcript, and for this reason, they are useful for long mRNAs and transcripts with significant secondary structure. Typically 6-mers are used but use of 8- or 9-mers may facilitate synthesis of longer cDNAs since they hybridize less frequently. Gene-specific primers often are used in one-step (coupled) RT-PCR. These enhance sensitivity by directing all RT activity to a specific message as opposed to transcribing the entire RNA.

For RT-qPCR applications where the length of the target amplicon is about 100bp or less, using higher concentrations of random primers may be advantageous. You will have a higher probability of a priming event at the 3´ end of the mRNA, a greater chance of multiple cDNAs produced from each transcript and a higher probability of priming rare transcripts. Regardless of primer choice, the final primer concentration should be optimized. Design of primers spanning intron or intron-exon boundaries can insure that cDNAs are synthesized from spliced mRNA sequences and not the parent gene sequence in the gDNA.

Primers with modifications such as biotinylation or addition of sequence tags to the 5´ end may be useful in purification and/or analysis of cDNAs synthesized from specific sense or anti-sense template strands (Haist et al., 2015; Kawakami et al., 2011; Feng et al., 2012). Such strategies can be a powerful tool in studying bi-directional mRNA synthesis in eukaryotic cells.

Secondary Structure of RNA

RNA molecules have secondary structures that can affect transcription, and high GC content can reduce the efficiency of reverse transcription. High-temperature denaturation before transcription or synthesis at temperatures higher than those used in standard RT reactions, may be beneficial with difficult templates. Difficulty in separating double stranded structures can be overcome by five-minute denaturation at 65°C, which relaxes the RNA. RT enzymes that are efficient at moving through secondary structures and GC-rich elements at standard temperatures (37–42°C) are commercially available.

cDNA quantification by qPCR

Heat inactivating the reverse transcription reaction following cDNA synthesis is highly recommended. After RT inactivation, either proceed directly to target-specific quantitative analysis of the cDNA by qPCR or freeze the inactivated reactions for storage until they are needed. qPCR reaction mixes typically accommodate the addition of cDNA sample volumes representing up to 20% of the total reaction volume.

The cDNA may be added directly to the qPCR, as undiluted reverse transcription reaction product, or it may be diluted. The dilution factor must be experimentally determined to be appropriate for the amount of RNA template mass and proportional reverse-transcript representation in the cDNA sample. Quantification of cDNA is critical for success of RT-qPCR workflow. Generally, for cDNA quantitation using the default analysis settings of many real-time instruments, the amount of cDNA used as template should not exceed the quantity that would be obtained by transcribing 100ng of input total RNA. The cDNA generated from highly abundant transcripts can be detected in less than 1pg of total RNA.

In application of all of these principles, the MIQE guidelines provide a basis for insuring that all critical sample quality and experimental design features are appropriately addressed and recorded.

Section 4

Recent Advances

Although reverse transcription has been a mainstream method for many years, innovative approaches and new applications continue to appear. These include improvements associated with use of thermostable reverse transcriptases obtained from novel sources or by screening mutants of mainstream RTs such as M-MLV (Arezi and Hogrefe, 2009; Mohr et al., 2015). Such polymerases may provide the ability to perform reverse transcription at 50oC or above, which could provide benefits for applications such as RNA-Seq by facilitating synthesis of longer cDNAs. A number of RT mutants are commercially available including M-MLV RTs that lack a functional RNAse H domain, which can add greater stability of the RNA template during reverse transcription.

Advances have also been made in the application of new technologies, such as Droplet Digital PCR (ddPCR) (Courdray and Neunier, 2015; Racki et al., 2014). Unlike traditional RT-qPCR, RT-ddPCR does not require the use of a standard curve, which makes it an attractive strategy for mitigating the effects of PCR inhibitors. Such inhibitors can have major impacts on standard curves required in typical RT-qPCR analyses.

Single-cell RNA-Seq is also becoming more prevalent (Saliba et al., 2014). Generally, the first step in RNA-Seq is to convert the poly(A)+ component of an RNA population to cDNA after which the cDNAs are subjected to next-generation sequencing. Ideally, this RT step will be both efficient and non-biased which may require novel strategies, such as creation of a single-cell cDNA library immobilized on beads (Huang, et al., 2013).

Section 5

Conclusion

Reverse transcription is a critical step in RT-qPCR and a wide range of other applications performed routinely in laboratories around the world. However, despite its centrality in molecular biology research, the critical nature of the RT step as a factor determining the overall quality and validity of results may be underappreciated. RNA template quality and experimental design details must be carefully considered and performance characteristics such as RT efficiency and extent of RT bias must be clearly understood. The MIQE guidelines – Minimum Information for Publication of Quantitative Real-Time PCR Experiments, provide a framework which, if adhered to, ensures that critical experimental design and performance details are captured.. In addition to guiding individual researchers towards more effective experimentation, the MIQE guidelines foster consistency across laboratories and facilitate appropriate and effective assessment of experimental results reported in the scientific literature. Adherence to these guidelines becomes even more necessary as innovation in the development of new enzymatic tools and novel applications in the field of reverse transcription continues.

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Chapter 5

qPCR Oligonucleotide Synthesis and Design

At the interface of chemistry and biology

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Section 1

Introduction

Nucleic acid is the class of biomolecules comprised of DNA and RNA (Chargaff and Davidson, 1955). It is considered the original organic compound from which all life originated, and provides the molecular blueprint for every organism on earth. Genetic information is encoded within a sequence of nucleobases that hydrogen bond of those of a partner strand, interlocking with remarkable precision in the form of a double-helix. The specificity of this base-pairing enables DNA to store, retrieve, and replicate genetic information for which it is best known, but also provides emergent properties that are not so easily predicted. Nucleic acid catalyzes certain chemical reactions (Hammann and Westhof, 2007) and entwines higher order structures—molecular machines that assemble the building blocks of the cell.

Oligonucleotides are fragments of DNA that represent critical components for all manner of biological study. A short oligo can selectively bind its complement from within the billions of bases comprising a genome, and this recognition provides the basis for many genetic methods (Saiki et al., 1988). The sensitivity and specificity of such assays are fundamentally influenced by constituent oligos, and so selecting their sequences requires careful consideration, accompanied by meticulous handling and use. Most oligos are synthesized chemically, by virtue of an industrial manufacturing process that differs significantly from the research environment in which they will be utilized. It is therefore important to understand

the chemical synthesis of oligos to better appreciate their application.

The facile manufacture of short oligo sequences made possible the invention of a remarkable technique for enriching DNA in a test tube: the polymerase chain reaction. PCR quickly inspired a revolution in molecular biology, allowing for whole genes to be amplified and then isolated with ease. A subsequent adaptation of this fundamental technique is quantitative PCR (qPCR) where the sequence enrichment is monitored in real-time, and the kinetics of amplification analyzed to gauge the quantity of that sequence prior to the reaction. Quantitative PCR is a powerful technique for gene expression analysis: measuring the up- or down-regulation of genes based on their transcript levels. Anything with a genetic element can be revealed through qPCR, including the presence of cancer or infectious disease, and the prevalence thereof. Commercial processes use PCR to screen for genetic modification within the agricultural industry (Broeders et al., 2012; Mbongolo Mbella et al., 2011) and monitor for spoilage microbes in food production (Hoorfar, 2011; Postollec et al., 2011). The technique is widespread in medical diagnostics (Mackay et al., 2002; Valones et al., 2009), forensic testing (Huijsmans et al., 2007; Maeda et al., 2010; Vennemann and Koppelkamm, 2010), basic research (Deepak et al., 2007), and even integrated into secondary school science curriculums (Hamilton et al., 2006). While qPCR testing has become ubiquitous there is little consensus on the design of oligos, and still need for uniform standards to report on the sequences, their modifications, and manufacture. We will explore each of the MIQE oligonucleotide standards (Bustin et al., 2009; Taylor et al., 2010) (Table 4.1) for proper reporting of experimental results, while covering the fundamentals of oligo synthesis and design.

Oligonucleotide Information	Reporting Requirement
Primer sequences	**Essential**
Primer database identification number	Desired
Probe sequence with detection format	Desired (when available)
Location and identity of oligonucleotide modifications	**Essential**
Manufacturer of oligonucleotides	Desired
Oligonucleotide purification method	Desired

Table 4.1 - MIQE qPCR oligonucleotide information categories and the importance of reporting these requirements for MIQE compliance

Section 2

Oligonucleotide Manufacture

1. Oligo Synthesis
2. Oligo Purification

Oligo Synthesis

Oligos were not always so readily synthesized. In 1980, the manufacture of a 15-base sequence cost US $9,000 for 100 nmol delivered at 95% purity. Today this oligo would cost at least 100-fold less. The fundamental difference is that oligos were originally made using solution-phase chemistry (Khorana, 1968), with many different byproducts generated in unison. From that milieu the desired product was isolated and enriched. Modern oligos are now made through solid-phase synthesis, growing strands bound to an immobile support (Figure 4.1). Computers dispense reagents through a porous matrix representing the site of reaction, and excess reagents are washed through so that the cycle can begin anew. These collective improvements allow for each base to be coupled in series in a much more deterministic fashion.

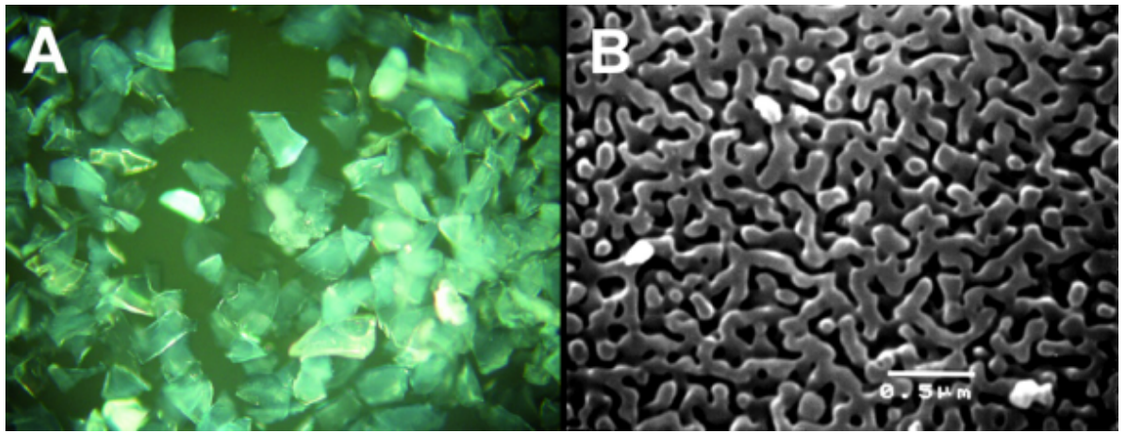
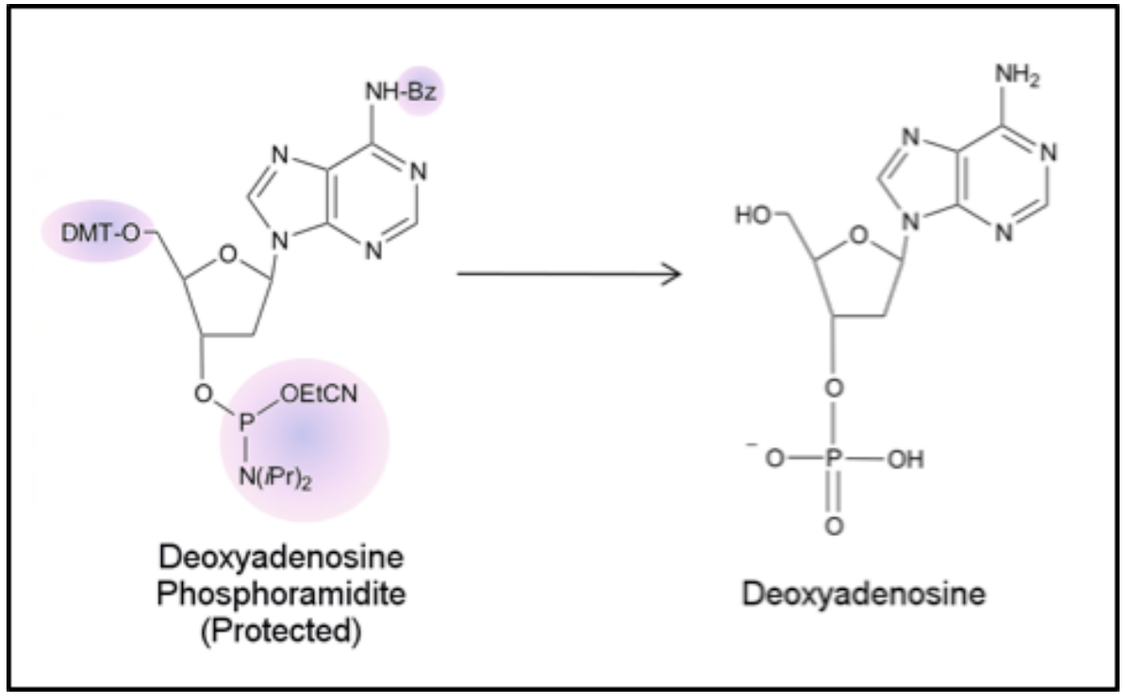


Figure 4.1 – Controlled pore glass (CPG) represents a solid support for oligo synthesis. CPG is created from powdered glass particles (A) that are etched with pores to provide a large surface area for chemical activation (B). The CPG is then functionalized with the attachment of the first base typically representing the 3' terminus of the oligo.

Each base is incorporated as a phosphoramidite precursor (Figure 4.2), representing the culmination of many important improvements by celebrated chemists like Khorana (Khorana, 1968), Letsinger (Letsinger and Ogilvie, 1969), and Caruthers (Caruthers, et al., 1983). Nucleoside phosphoramidites differ fundamentally from the dNTPs found in nature but the principle is the same: a reactive precursor ready for incorporation. Phosphoramidites are responsible for the spectacular efficiency of chemical synthesis but are so reactive they can decompose upon exposure to water. Oligonucleotide manufacturing is therefore conducted entirely within organic solvents, kept anhydrous through desiccants and discipline.

Figure 4.2 – Phosphoramidites - the Building Blocks for Oligo Synthesis



Deoxyadenosine phosphoramidite, with reactive sites and protecting groups highlighted in purple (shown at left). After synthesis is complete the remaining protecting groups are removed resulting in the final nucleotide (shown at right).

The cycle of chemical reactions is also quite different from their enzymatic assembly *in vivo*, yet the final product is indistinguishable from its natural archetype.

Figure 4.3 – Phosphoramidite Oligo Synthesis

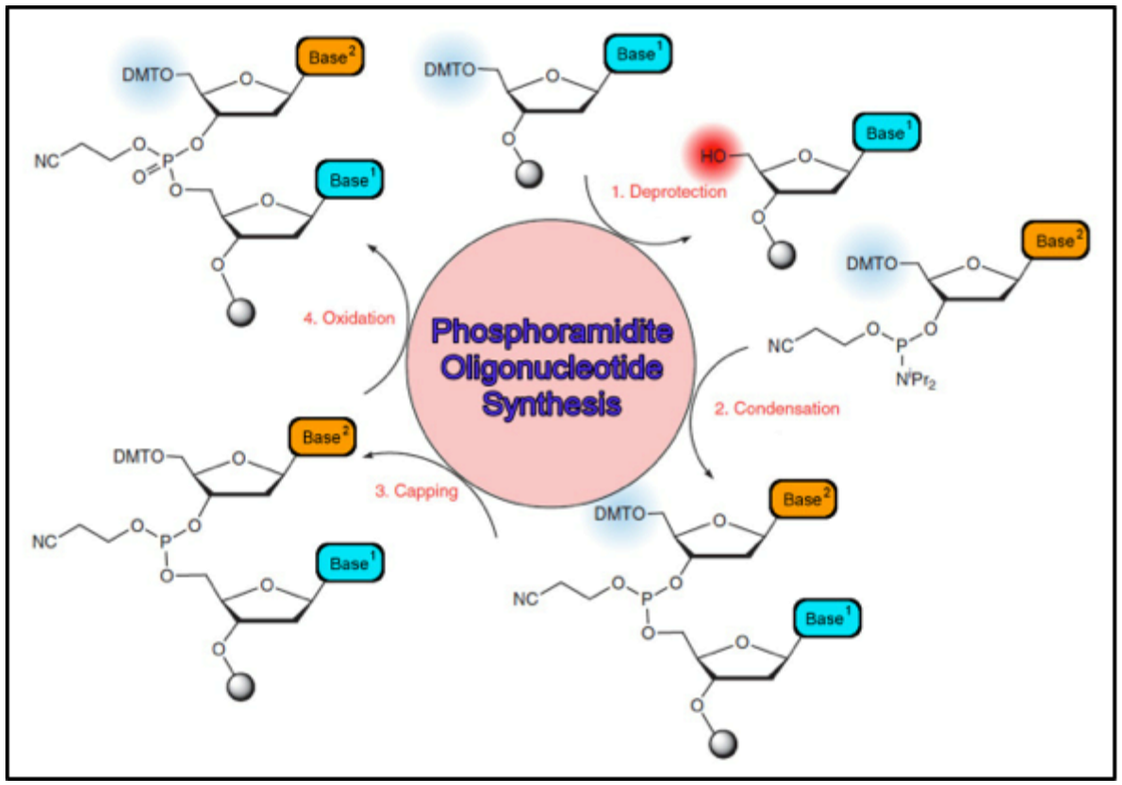


Diagram of oligo synthesis with the phosphoramidite method, culminating in the addition of a single nucleotide. The cycle is repeated for each subsequent base.

The reaction proceeds through a cycle of four basic steps (Figure 4.3):

(1) Deprotection: The synthesis cycle begins with the removal of the dimethoxytrityl (DMT) protecting group on the nucleotide bound to the solid support, to present a functional site for the next amidite to react. This DMT group is removed under acidic conditions, typically by treatment with dichloroacetic acid (DCA).

(2) Condensation: The amidite formulation of the next base is introduced into the reaction vessel and coupled to the deprotected hydroxyl using a tetrazole activator to form a phosphite triester linkage between the two nucleosides. There will always be a small percentage of bases that are left unconjugated and with a hydroxyl that remains reactive. During later cycles, coupling an amidite to those residual sites would skip a base and produce an oligo with the wrong sequence. These synthesis errors are known as a deletion sequence (e.g. “N-1 failure”).

(3) Capping: Any residual nucleotides with free hydroxyls are prevented from further reaction by the addition of an acetyl group. The majority of oligos that had successfully coupled are already protected by a DMT on the 5’ OH of their newly incorporated nucleoside and so capping leaves them unaffected.

(4) Oxidation: The phosphite triester linkage is oxidized with iodine treatment to convert it to a phosphate and complete the

cycle. This process of adding a single base to the strand proceeds with remarkable efficiency, typically above 99% which is fundamental to achieve longer sequences with minimal truncated byproducts.

(5) Final work-up: After repeating the cycle for each subsequent base, the final oligo still presents an assortment of protecting groups: cyanoethyls on the phosphate linkages and blocking groups on exocyclic amines (Figure 4.2). These groups are removed through a final deprotection treatment under basic conditions, typically heated ammonia. A base-labile linkage connects the oligo to the solid support, and so this process simultaneously releases the newly formed oligo into solution. The finished oligo is eluted from the column and ready for further manipulation (Blackburn, 2006; Kates and Albericio, 2000).

The development of oligonucleotide synthesis chemistry and its subsequent automation played a pivotal role in the invention of PCR. In the early 1980's Biosearch, Inc. developed an instrument with microprocessor-controlled reaction vessels representing a seminal DNA synthesizer—the first commercially successful model instrumentation. Kary Mullis used that very synthesizer in 1983 to produce his first oligo primers, and demonstrate a concept he called the polymerase chain reaction (Figure 4.4). For that invention he was later given the Nobel Prize in 1993, and acknowledged the contribution of Biosearch's device in his acceptance speech (Mullis, 1994). Every PCR reaction requires primers, and so oligos became critical components for all manner of investigations into the genome. Recombinant DNA technology, genome sequencing, and molecular diagnostics were all made practical through synthetic oligos and PCR.

Figure 4.4 – The beginnings of PCR

Picture of Kary Mullis accepting the Nobel Prize in 1993 for the invention of PCR (left). Shown on the right is the first commercial DNA synthesizer, Biosearch's SAM I, which Mullis used to produce the first PCR primers. Mullis later autographed one of these original devices which resides at Biosearch's headquarters in Northern California.

Dedicated synthesis facilities now have robotic platforms fed by process piping to rapidly produce oligos with little need for human intervention (Figure 4.5). Industrial DNA synthesis allows a researcher to receive novel oligo sequences of their own design, all within a manner of days. However, with the convenience of

commerce in the Internet Age, there is risk of overlooking the principles of their chemical synthesis, those that dictate fundamental limitations and also an enormous range of possibilities.

Figure 4.5 – Modern DNA Synthesis Facility

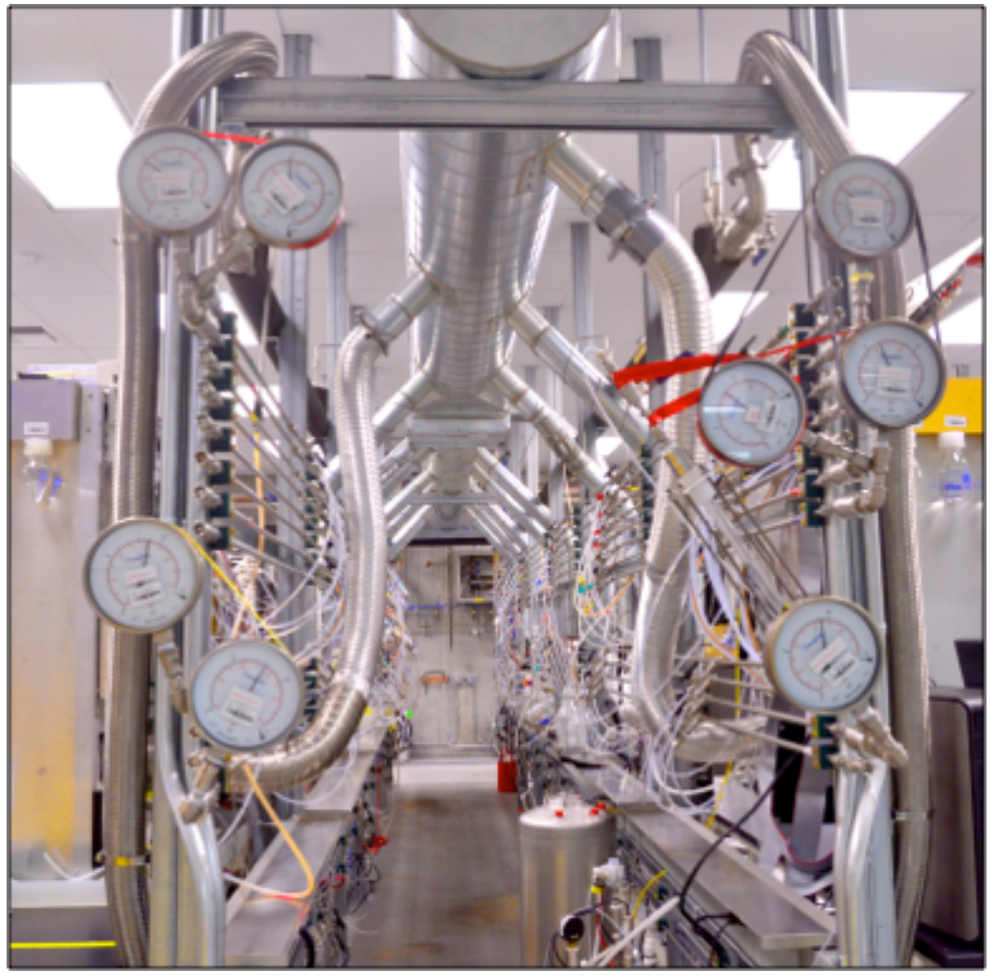
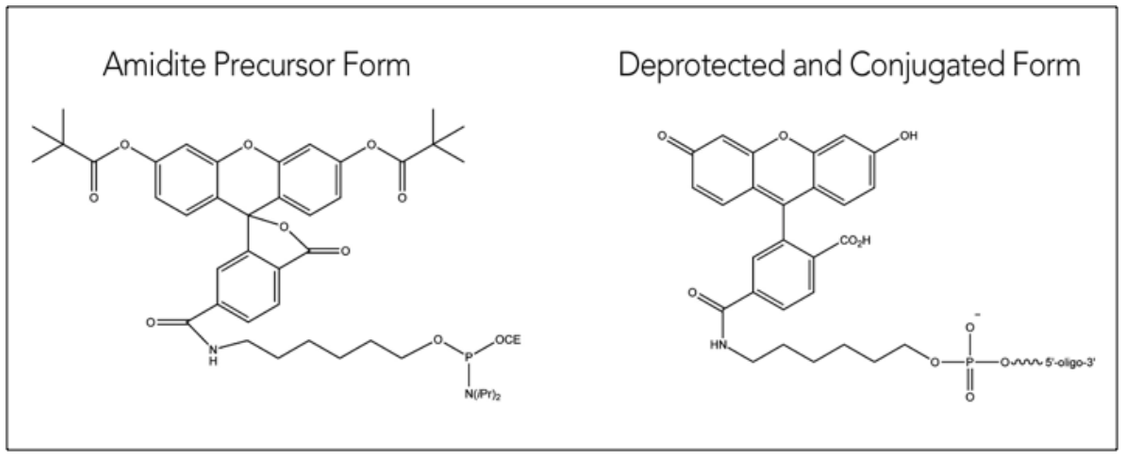


Image of a modern oligonucleotide processing plant. The image shown here displays the process piping that delivers to many automated synthesizers the phosphoramidite formulations of the standard bases (A, T, C and G) as well as the ancillary reagents, for a highly efficient manufacturing process.

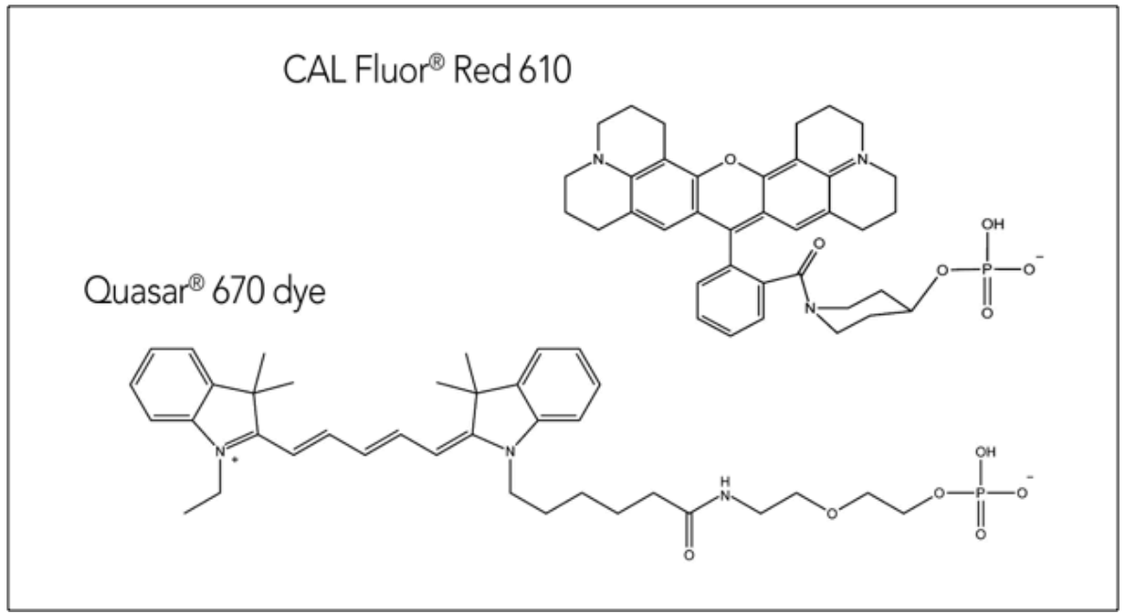
A synthesized oligo of 15 bases has over a billion possible sequences when composed of the standard A, T, G, and C. The possibilities are not limited to the four canonical nucleotides, but include other natural nucleotides like uracil and artificial bases with unusual hydrogen bonding like isoguanine. Modifications entirely unrelated to nucleic acid are also incorporated directly into the oligo strand: fluorophores, linkages, and other moieties that impart novel properties far beyond its behavior as a biomolecule (Figures 4.6 - 4.8). These labels may also be formulated as phosphoramidites for automated incorporation during oligo synthesis.

Figure 4.6 – Fluorescein (FAM)



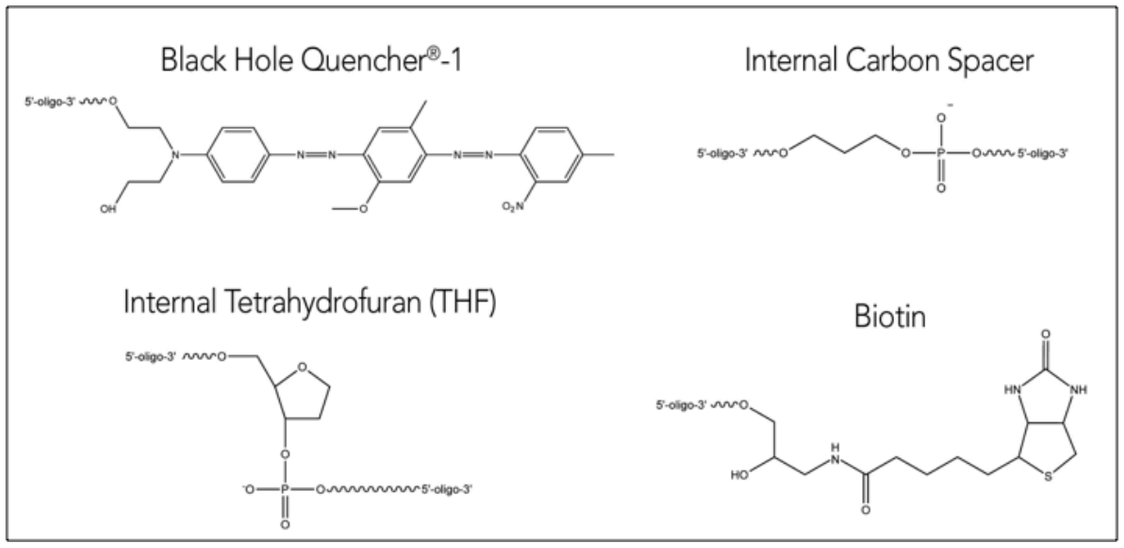
Fluorescein amidite (FAM) is shown at left with pivaloyl groups protecting reactive hydroxyls on the chromophore. Conjugation and deprotection reveals the final fluorescent label, at right, as an oligo modification on the 5' terminus. Fluorescein represents the quintessential reporter for all manner of genetic methods including sequencing, fragment analysis, and qPCR.

Figure 4.7 – Longer Wavelength Fluorescent Modifications



Other reporters for oligo conjugation include xanthene-based fluorophores like CAL Fluor® Red 610, and cyanine-based fluorophores like Quasar® 670. They can be used in combination with FAM and yet detected independently, since they emit maximally at different wavelengths designated by their titles.

Figure 4.8 – Additional Examples of Oligo Modifications



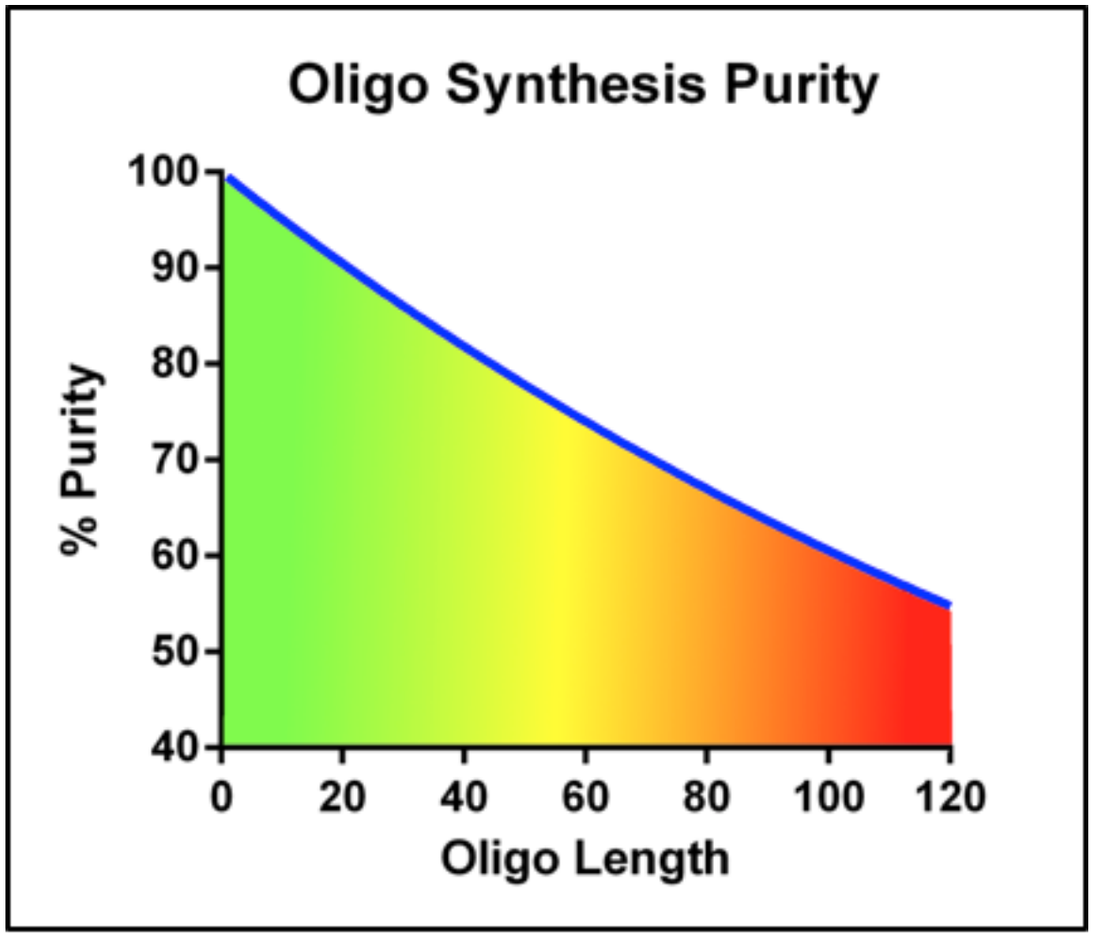
Oligo modifications are not limited to fluorophores. Top-Left: The Black Hole Quencher®-1 Dye is a chromophore designed to extinguish the signal from a reporter such as FAM. Top-Right: A short aliphatic "spacer" is designed to disrupt the sugar-phosphate backbone. Bottom-Left: A tetrahydrofuran linkage represents deoxyribose without a base moiety. Bottom-Right: A biotin modification, often used as an affinity label to bind streptavidin-coated beads or solid supports.

Each modification changes the oligo behavior in a predictable way. Multiple modifications are designed to interact with each other when tethered through the same strand. They are used to report on molecular interactions like binding events or changes in conformation – producing a signal that is measureable on a macroscopic scale (Figure 4.6 and 4.7). The wealth of potential modifications gives rise to oligo constructs suited for a range of molecular applications (Figure 4.8). Modern oligos are thus an artifice, emerging from computer-controlled reactors rather than a living cell, and the iconic double-helix is transforming into something much more.

Oligo Purification

The chemical synthesis of oligos yields a crude product containing impurities and truncated oligos that are a natural byproduct of the manufacturing process. Different contaminants potentially affect functionality depending on the oligo type and application. In particular, the longer the oligo, the higher proportion of failure sequences that contaminate the prep. All else being equal, a 100-mer with a cycle efficiency of 99.5% is only 60% pure (0.995 raised to the power of 99), while a shorter 20 base oligo would be closer to 90% pure (0.99519) (Figure 4.9).

Figure 4.9 – Oligonucleotide Synthesis Efficiency



Assuming a coupling efficiency of 99.5% efficiency per reaction cycle, the purity of the final product will decrease with increasing sequence length. Purification is necessary to enrich the full-length product.

For typical PCR primers 15-25 bases in length, the truncated oligos may not affect the overall PCR performance as they comprise a small fraction of the finished product. Oligos for use in medical diagnostics require optimal purity to ensure minimal variation in manufacture, irrespective of the functional impact of contaminating byproducts. Modified oligos introduce new categories of contaminants into the production process: fluorescent-labeled oligos can have residual fluorophore left unconjugated that would elevate the background signal in the assay (Figure 4.10) (Yeung et al., 2004). Chromophores and other modifications are naturally sensitive to the conditions of DNA synthesis and so their degradation products must be removed. Considering the potential impact to oligo functionality, it is very prudent to report in publications the purification regime used in the manufacture of each oligo. Common purification methods are as follows:

Salt-free – Desalting is done to remove excess ammonium ions (if any) and the free protecting groups now in the form of organic salts. However, some cations must remain coordinated to the negatively-charged phosphates in the oligo. Certain applications might require salt exchange to replace with another counter-ion like Na+. This process will not remove aberrant sequences generated during manufacture and so the product remains unpurified (crude).

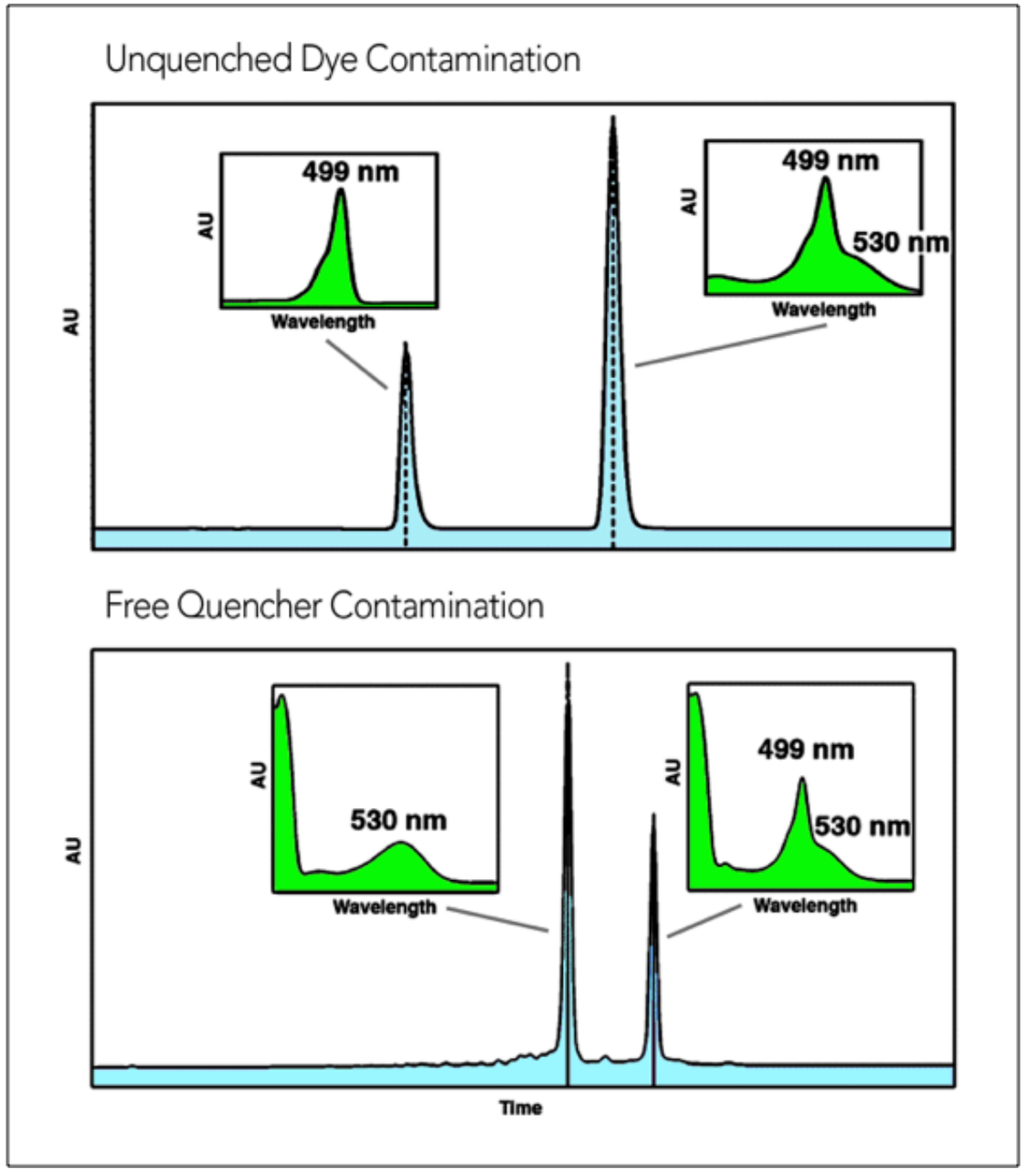
Reverse Phase Cartridge (RPC) – This method of purification is appropriate for unlabeled oligonucleotides, such as standard qPCR primers. The principle of reverse phase chromatography is

used to bind the full-length product to the cartridge, which has a hydrophobic affinity through its terminal DMT protecting group left attached. Truncated sequences that do not present this moiety are eluted through, after which the DMT group on the desired product is removed.

Reverse Phase HPLC (RP-HPLC) – Reverse phase high-performance liquid chromatography also separates the synthesis products according to their hydrophobic affinity. This purification method is well-suited for fluorescent-labeled oligos since organic dyes are usually hydrophobic in character. The full-length product will elute at a different interval than the byproducts, which allows for the removal of fluorescent contaminants (Figure 10).

Anion Exchange HPLC (AX-HPLC) – This method of purification separates the products of oligo synthesis according to their charge. Truncated oligos resulting from a coupling failure will have fewer phosphate groups and so elute at a different interval than the full-length product. AX-HPLC is appropriate for unlabeled oligos and those dye-labeled oligos modified on their 3' terminus, whose failure sequences may resolve poorly by RP-HPLC.

Figure 4.10 – Reverse Phase and Anion Exchange HPLC



Top: RP-HPLC trace of a crude FAM-BHQ1 probe harboring a contaminating byproduct labeled only with FAM. Bottom: AX-HPLC trace of a crude FAM-BHQ1 probe and contaminant

labeled only with BHQ1. The absorption spectra of each fraction are used to characterize the species within.

Dual HPLC (AX/RP-HPLC) – This method is the sequential application of anion exchange followed by reverse phase HPLC. The combination of the two purification regimes separates different categories of contaminants to achieve a very high level of purity. Note that as the stringency of purification increases for an oligo, the overall yield of final product naturally decreases for a given synthesis scale.

PAGE – Polyacrylamide gel electrophoresis (PAGE) is a method to resolve nucleic acid strands according to their charge and molecular weight, and then isolate the desired band. This method provides excellent resolution of the full-length product according to the number of bases, but is relatively laborious. PAGE purification is particularly well-suited to longer oligonucleotides greater than 50 bases long that require a high standard of purity, but is restricted to sub-micromole quantities of crude oligo.

Section 3

Principles of Oligo Design

1. PCR Primers
2. Melting temperature
3. DNA Binding Dyes
4. Fluorogenic probes
5. Assay Design Software

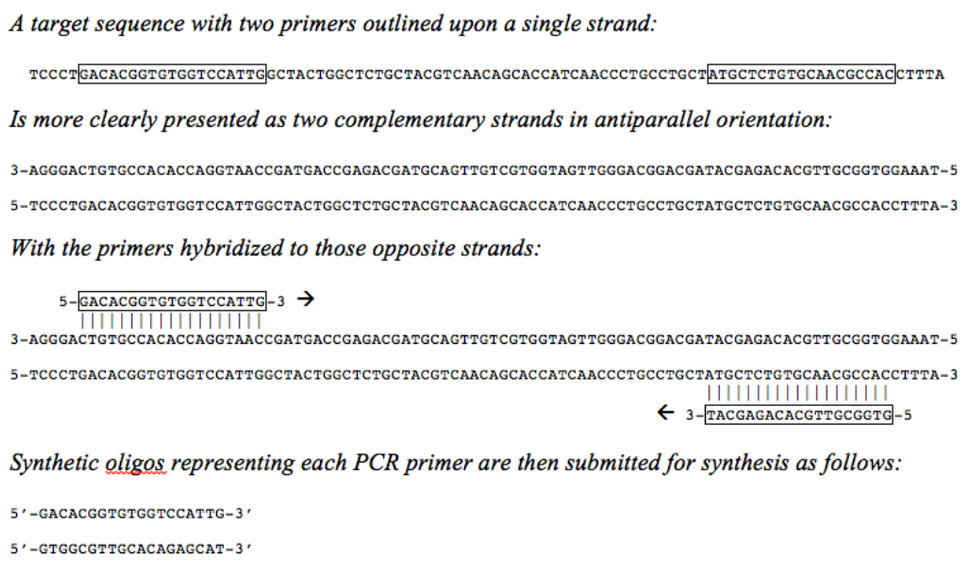
Oligo design is informed by an ensemble of rules and guidelines, even myth. Certain aspects of the PCR process remain elusive despite decades of the method's application and optimization, which testifies to the magic of a chain reaction triggered by a molecular binding event. The random encounters of individual molecules invites some conjecture, yet these earliest cycles of amplification have pronounced effect on reaction kinetics and the composition of product.

PCR Primers

A critical component of any and every PCR reaction is the primer-set. These oligos are selected to flank the sequence of interest and so delineate the target for replication. They trigger polymerase extension upon binding to DNA, and are designed from opposing strands to facilitate exponential amplification—the hallmark of PCR. Complementary strands bind one another in antiparallel orientation, according to the 5' and 3' positions on the ribose ring. DNA Polymerase synthesizes the new strand from the 5' end toward the 3' end. Similarly, sequences are written in 5 to 3 direction by convention, and is the assumed orientation whenever the polarity is not annotated on sequence ends. This convention also applies to synthetic oligos: a 5 to 3 direction is used to request primers for manufacture. One must be mindful of the duplex nature of DNA, and the antiparallel orientation of each strand. For simplicity of presentation,

software programs will commonly highlight both binding sites upon a single sequence, which can be misleading (Figure 4.11).

Figure 4.11 – Orientation and Annotation of Oligo Sequences



A primer-set is designed such that each oligo binds to the opposite strand, even when both are highlighted upon the same sequence for a more compact presentation. The direction of polymerization is indicated with arrows.

Given the importance of primers to the PCR reaction, it is essential to report the exact oligo sequences without ambiguity in their orientation. Additional information about the binding sites within the target sequence is ideal.

Primer sequences should be sufficiently unique to amplify the desired target to the exclusion of other sequences in the specimen or sample. Specificity is dictated by their sequence design, and also from both oligos acting in combination: they each bind to the same target region, within a reasonable distance of the other. The ideal range of this distance is dictated by the processivity of the enzyme and the specific PCR reaction conditions. It would seem highly unlikely for an unrelated target to harbor the same sequence(s) by coincidence, except biology does not always follow the rules of probability. Genomes are comprised of many repeated regions representing common motifs, gene families, transposable elements, and the relics of gene duplication or viral infection. Certain applications must distinguish sequences that differ by only one or a few base-pairs in a background of very similar DNA, including the detection of circulating tumor cells (Strati et al., 2013) and of fetal DNA in maternal blood (Papageorgiou and Patsalis, 2012). Such demanding specificity is made difficult from the fact that oligos may continue to bind a sequence that is not fully complementary. Specificity therefore requires consideration of both sequence identity and the thermodynamics of oligo hybridization.

Melting Temperature

PCR depends upon elevated temperatures to dissociate the target strands followed by reduced temperatures to bind the primers to their complementary sequences. The cadence of thermal cycling synchronizes each replication event, and so the thermodynamics of oligo hybridization is a guiding principle of primer design.

Many software programs model primer binding with a simplified two-state model: hybridized and unhybridized conformations that coexist in equilibrium. The temperature of melting, or TM, represents the threshold where both conformations are proportionate. Binding is energetically favored beneath the calculated TM and so those candidate sequences that register 5 to 10 ˚C above the annealing temperature of the reaction are normally advanced for further consideration as PCR primers.

Melting temperature is a convenient value, often the guiding principle for oligo design, but its limitations are easily overlooked. It is important to remember the oligo in solution does not bind at a precise temperature but rather a range of temperatures, with varying proportion bound across that range. The actual melting temperature can only be measured empirically as it depends on the oligo concentration, buffer, salts, etc. (von Ahsen et al., 2001). A calculated TM is therefore a prediction, with different software programs outputting values that vary as much as 10 ˚C! (Chavali et al., 2005; Panjkovich and Melo, 2005). These discrepancies emerge from assumptions regarding reaction components, and also from fundamentally different algorithms. Design rules were established during the infancy of qPCR, when TM's were generally calculated using the Breslauer nearest neighbor thermodynamic values published in 1986 (Breslauer et al., 1986). More accurate values were measured and published in 1998 as the "unified" nearest neighbor thermodynamic values (SantaLucia, 1998; Watkins and SantaLucia, 2005), and yet many legacy programs continue to rely upon the dated values. Given all these discrepancies, TM is better viewed as a general guideline rather than a rigid rule. It is only one of many parameters that inform assay design, that all vary somewhat according to the assay format.

DNA Binding Dyes

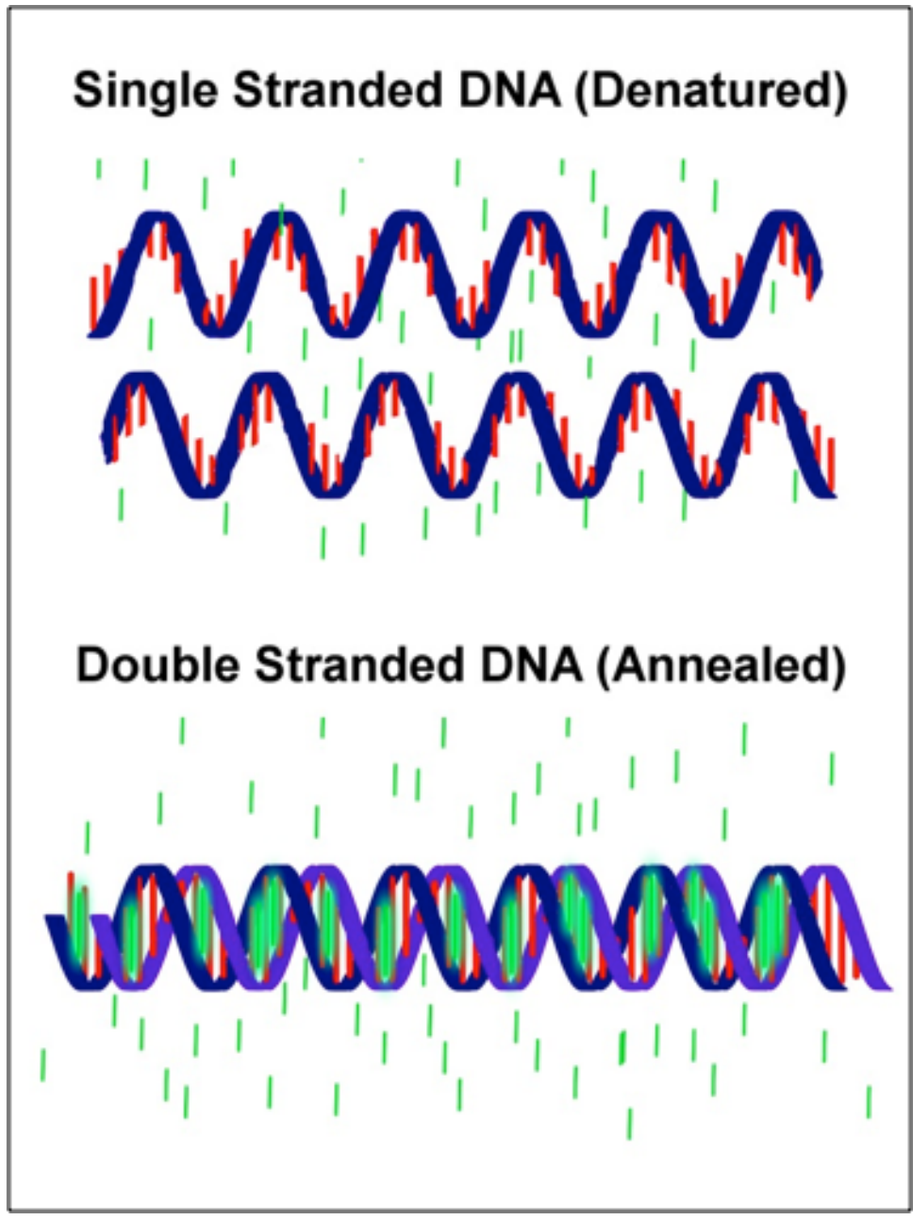
Real-Time PCR is the ability to monitor the PCR reaction in real time, during the amplification event itself. This technique requires a detection method to reveal the accumulation of product within the reaction. Dyes that fluoresce upon binding duplex DNA are a sensible solution (Figure 4.12). Such fluorophores have been used for decades to visualize PCR products following amplification, usually in combination with gel electrophoresis to isolate and manipulate the desired product. The archetype DNA binding dye is 2, 7-diamino, 9-phenylphenanthridinium 10-ethyl bromide, more commonly known as ethidium bromide. This molecule is only weakly fluorescent until intercalating between the

bases of duplex DNA, where it becomes protected from the surrounding solution. Upon illumination with ultraviolet light the complex is made to glow, with bands corresponding to DNA fragments visible to the human eye (Aaij and Borst, 1972; Borst, 2005; LePecq and Paoletti, 1967; Sharp et al., 1973; Wahl et al., 1970; Waring, 1965). Notably, Jean-Bernard Le Peq correctly predicted that ethidium bromide could be "applied to the study of DNA polymerase in action" (Le Pecq, 1971).

In the early 90's Russell Higuchi and colleagues did exactly that by spiking ethidium bromide into the PCR reaction prior to thermal cycling rather than afterwards (Higuchi et al., 1992). Doing so creates a homogeneous system in which the reaction vessel need not be opened for visualization. Instrument optics tuned to the right wavelengths can detect fluorescence through the tube without further manipulation.

Subsequent dyes were developed with improved properties including SYBR Green 1 (Schneeberger et al., 1995) which has a 1000% increase in fluorescent emission when bound to double-stranded DNA (Dragan et al., 2012; Zipper et al., 2004). More recent dyes include EvaGreen (Mao et al., 2007), LC Green (Wittwer et al., 2003), ResoLight (Schütz and von Ahsen, 2009), and BEBO (Bengtsson, 2003). All of these dyes operate in a similar principal to ethidium bromide – increased fluorescence upon intercalation into double-stranded DNA.

Figure 4.12 – DNA intercalating Dyes.



Double-stranded DNA intercalating dyes – Dye molecules (green) are present in excess and diffuse free in solution with minimal fluorescence emission. When the dye comes in contact with dsDNA it intercalates between the stacked bases and assumes a

conformation that emits fluorescence at a particular wavelength upon excitation.

The advantages of a dsDNA binding dyes for qPCR are simplicity of design and economical assay development. However, this convenience comes at the expense of specificity. The dye molecule does not inherently distinguish between the target of interest and off-target amplification. PCR can generate spurious reactions, whether from mis-priming similar sequences, from other DNA fragments behaving as primers, or from primers binding themselves to form primer-dimers (SantaLucia, 2007). Unanticipated interactions compete with the intended reaction to produce multiple products that can swamp the target of interest. DNA binding dyes signal in response to any double-stranded product and so may report amplification even when the target is absent. False-positives are difficult to distinguish from a true result.

A melting curve at the end of thermal cycling is commonly used to gauge the specificity and composition of PCR product(s) with a DNA binding dye. Fluorescence is monitored while the temperature is slowly increased, to reveal different amplicons that dissociate at different intervals according to their fragment length and sequence composition. Dye fluorescence is extinguished when the two strands melt apart, and so the signal from the reaction vessel changes more abruptly at these transition temperatures. Melting points are more clearly presented using a first derivative plot of the signal intensity with respect to temperature. End-point analysis requires careful interpretation of the melt-curve in order to gauge reaction specificity—to distinguish the intended target from false products.

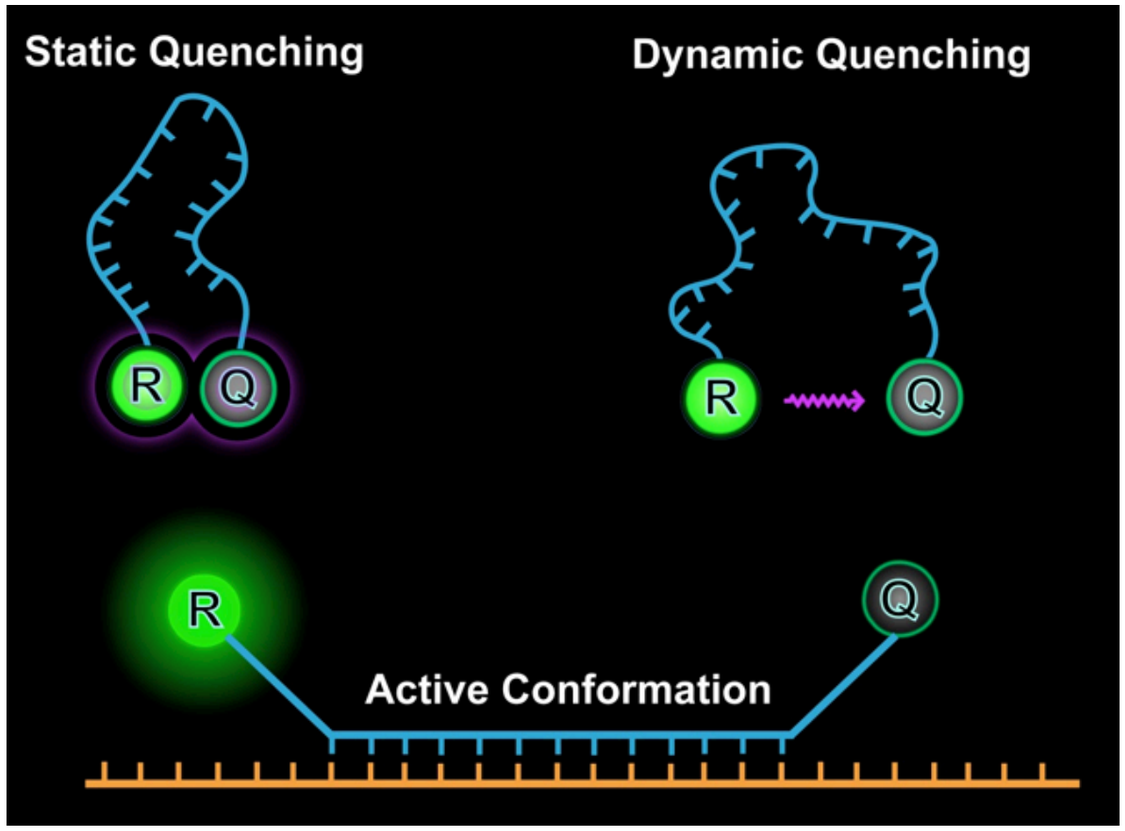
Fluorogenic Probes

Signal fidelity is improved through the use of fluorogenic probes —oligos designed to release fluorescence upon binding the target sequence. Probes hybridize between the primers, and so provide the assay with a third layer of specificity. While PCR primers may amplify off-target, a probe's signaling mechanism requires sequence recognition and so spurious products are less likely to generate a false positive result. Fluorogenic probes are ubiquitous in molecular diagnostics where specificity is an absolute priority.

Many different probe-types have been commercialized over the years, including Molecular Beacons (Tyagi and Kramer, 1996), TaqMan® Probes (Holland et al., 1991), and Scorpion® Probes (Whitcombe et al., 1999), to name a few. They differ in their sequence geometry and binding mechanism, but nearly all share a fluorescent reporter partnered with a quencher (Didenko, 2001; Ranasinghe and Brown, 2005). The quencher is a second modification to the oligo—a chromophore designed to absorb the signal from the fluorescent reporter when the two labels are in close proximity. By tethering both modifications to the same

oligo the dyes may interact through either Förster Resonance Energy Transfer (FRET) or else static quenching, where they contact one another to form a ground-state complex that is non-fluorescent (Johansson, 2006; Marras et al., 2002) (Figure 4.13). The duplex formed upon oligo hybridization is quite rigid and so increases the effective length compared to single-stranded oligo, which is much more flexible. Binding of the probe to the target sequence is therefore sufficient to disrupt the interaction between dyes positioned at opposite ends of an oligo (Parkhurst and Parkhurst, 1995).

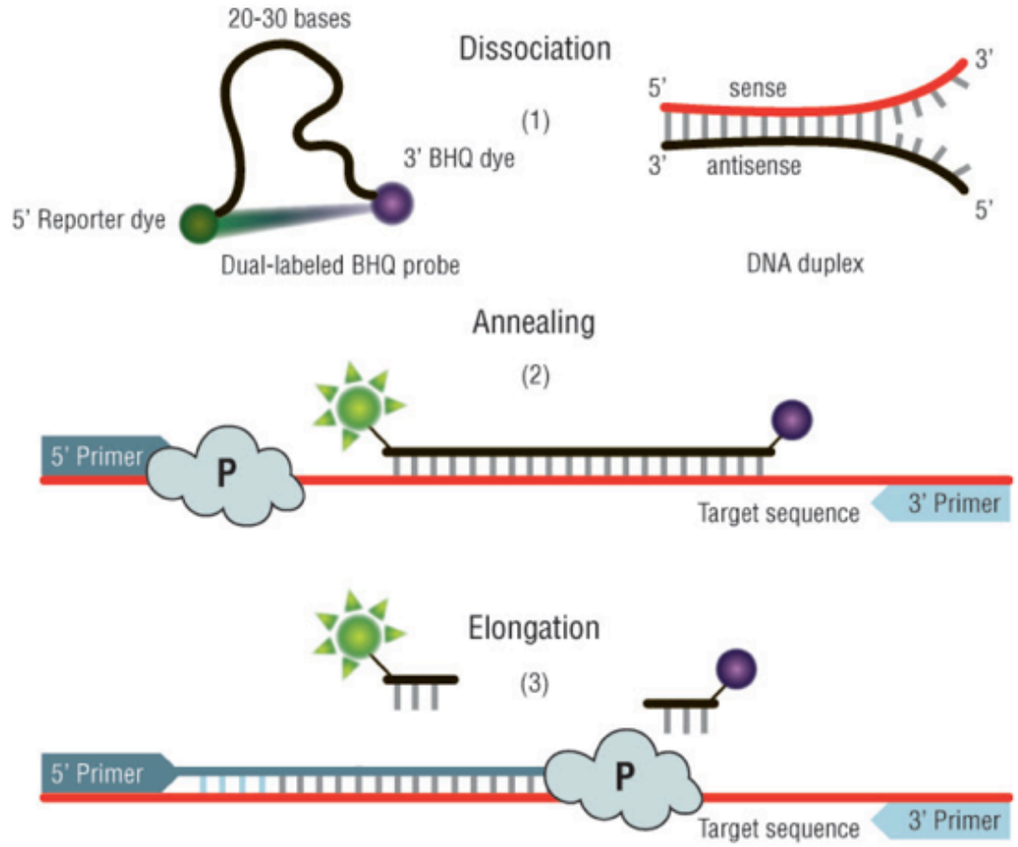
Figure 4.13 – Quenching Mechanisms in Dual-Labeled Probes



Quenching and Signaling Mechanisms of Fluorogenic Probes – Three conformations of a fluorogenic probe are presented. In either single-stranded form (top), the excitation energy of the reporter dye is transferred to the quencher preventing signal release. With static quenching, the reporter and quencher physically associate to form a non-fluorescent intramolecular dimer. In dynamic quenching, the reporter and quencher are in sufficient proximity to permit FRET quenching through space. Upon hybridizing to the target sequence the reporter and quencher are separated to allow fluorescence emission (bottom).

The signal release upon hybridization can be made permanent by hydrolyzing the oligo between the fluorophore and the quencher. In fact, cleavage is accomplished when Taq polymerase extends from a primer and encounters the probe in its path (Figure 4.14). The nuclease activity of the polymerase cleaves the probe one or several bases from its 5' end until the probe loses binding stability and is displaced from the strand. This signaling mechanism is the basis for hydrolysis (TaqMan®) probes, a commonly-used probe-type and the focus of our discussion regarding the parameters for probe design.

Figure 4.14 – Signaling Mechanism of Hydrolysis Probes



Mechanism of Hydrolysis Probes in qPCR – After denaturation, the probe and primers anneal to the dissociated strands. During elongation the polymerase extends from the primer, encounters the probe, and hydrolyzes nucleotide fragments that are displaced from the strand. Cleavage of the probe untethers the fluorophore and quencher to make signal release permanent.

The complete reaction mechanisms of various probe-types can be found as an animation on the following webpage:

Real-time PCR Probe Animations

To properly signal the amplification from each cycle, the probe must hybridize to the dissociated target before the primers replicate the strand and mask over the binding site. This is accomplished by designing the probe to bind with more stability than the primers. A common design guideline is to select probe sequences with a TM of 70 ˚C, elevated above that of the primers. Probes are typically designed 20 – 30 bases long in order to accomplish that TM, in the absence of additional modifications to increase binding stability. Target sequences that are particularly AT-rich might require longer sequences still, but those probes >30 bases can suffer from poor quenching efficiency. This is because FRET quenching diminishes quite rapidly with increasing separation between the dyes according to a relationship of (1/r)^6, where r is the distance through space (Cardullo et al., 1988). Single-stranded oligos are thought to behave as a random coil and so the effective distance is the average of many conformations, but the principle remains the same: increasing sequence length diminishes the quenching efficiency, resulting in probes with elevated baseline fluorescence and poor signal-to-noise values. Probe design is thus a careful compromise between longer sequences to promote binding stability and shorter sequences to promote quenching efficiency.

The length limitations governing probe design can be improved through certain modifications and design strategies. The quenching efficiency of longer probes can be improved by engineering secondary structure into the sequence itself. Molecular Beacons have self-complementary sequences on the 5’ and 3’ terminus that hybridize to one another to form a stem-loop conformation. This hairpin structure positions the reporter and quencher into immediate contact for remarkably efficient quenching. Alternately, the quencher can be positioned at an internal residue for closer proximity to the fluorescent reporter. However, the 3’ position now vacated must still be modified with a blocker such as a terminal phosphate or aliphatic carbon spacer, to prevent the probe from behaving as a primer and triggering extension.

A probe’s binding stability can be strengthened through a chemical moiety called Minor Groove Binder (MGB), used to modify either terminus (Kutyavin et al., 2000). The MGB allows for the design of shorter sequences that maintain the proper TM, as do non-standard bases including Locked Nucleic Acid (LNA) and propynyl residues used in BHQplus® probes. All of these modifications serve to increase the TM beyond what their sequences would indicate, but also require careful consideration of their unique contributions during the process of assay design. A sequence originally designed as an MGB probe may not function as a traditional probe, and vice versa. For this reason it is imperative to report the sequence of the probe design, and also

any modifications used in its manufacture. Chemical moieties designed to modulate the binding stability are often proprietary and so it is prudent to consult the recommendations of the oligo manufacturer for probe design, while citing that vendor in subsequent research publications. These vendors usually make available software tools to assist with sequence selection, based on design algorithms tuned for their particular chemistries. Such software programs consider not only the oligo length and melting temperature, but also hundreds of other factors that collectively improve the probability of success. We highlight some of these other parameters while introducing several software programs for assay design.

Assay design Software

Oligo sequences were originally selected by eye using simplistic TM estimations and anecdotal rules unique to each practitioner. Over time computation was brought to bear, to keep pace with the proliferation of genome sequences in public databases (Koressaar and Remm, 2007; Rozen and Skaletsky, 2000; Untergasser et al., 2007). Dedicated software is far more efficient than manual inspection to evaluate thousands of candidate sequences according to hundreds of parameters. High-throughput projects that target nearly every gene in the genome can only be accomplished through a batched design program, and particularly benefit from the consistency of a standardized design process combined with the efficiency of automated algorithms (Valenzuela et al., 2003). While not all are oriented for qPCR in particular, many software programs for oligo design are available for purchase. A representative selection of commercial programs include:

Oligo manufacturers also make available web-based tools for qPCR assay design, including:

Molecular Biology Insights	Oligo®
Life Technologies	Primer Express®
Premier Biosoft	Beacon Designer™
DNA Software™	Visual OMP™
Biosearch Technologies Inc.	RealTimeDesign™ Software
IDT®	SciTools®
Sigma-Aldrich	OligoArchitect™ Online
Life Technologies	Custom TaqMan® Assay Design Tool
Roche	Universal ProbeLibrary Assay Design Center

Figure 4.15 – Software Program for Primer and Probe Design

Screenshot from RealTimeDesign,™ Software showing oligo sequences and their properties. This program is available for free public use at: http://www.qpcrdesign.com

Beyond oligo length and melting temperature, computation is also used to evaluate numerous other characteristics such as particular sequence motifs or secondary structure in the target (Chavali et al., 2005; Rozen and Skaletsky, 2000; Untergasser et al., 2012). Target sequences that are particularly GC-rich may prove refractory to interrogation without the addition of denaturants into the reaction like DMSO and formamide. They may also harbor complementary regions that fold back on themselves to interfere with PCR (SantaLucia and Hicks, 2004). Folded conformations are particularly problematic with gene expression measurement since RNA forms stable hairpins that may resist reverse transcription. An equilibrium of secondary structures all compete with oligo binding, and best modeled using a partition function to compute the hybridization thermodynamics of the ensemble (Markham and Zuker, 2005; Mathews, 2006).

The base composition of the individual oligos is also consequential. It is widely understood to avoid probes with a 5' guanosine that may quench the reporter FAM, but other nucleotides also influence the spectral properties of proximal fluorophores (Marras et al., 2002). In general, G-rich oligos are problematic for reasons relating to both manufacture and functionality: a string of G's has the potential to collapse into a secondary structure called a G-tetrad, and associate into quadruplexes with other strands in solution (Burge et al., 2006). This feature may influence the binding behavior of the oligos and consequently should be avoided in primer and probe design.

Other bases that repeat four or more times are also problematic. These “runs” reduce sequence complexity and potentially cause mis-priming. The residues at the 3’ terminus of each primer have a pronounced influence on the assay performance. Some reports recommend a G or C as the final base to improve amplification efficiency, while others end their primers with adenosines to enhance priming specificity (Roux, 1995). Others still recommend to simply moderate the GC content in the final five bases (Nolan et al., 2006).

Complementary regions within and between the oligos may produce hairpins or dimers (Figure 4.16). It is therefore necessary to screen for these misalignments, particularly those that hybridize at the 3’ terminus of a primer. A run of complementary base-pairs may form a duplex that is capable of triggering extension, but even those alignments interrupted by mismatches deserve some consideration. Considering all the permutations of the oligos in the reaction this task is accomplished most efficiently through computation.

Figure 4.16 – Oligo Misalignments

```
Duplex: 4
   5' GCTTCCCATCCAGCTAGAGTC
                    ||||
   3'          CTGAGATCGACCTACCCTTCG

Match Count: 8
   5' GCTTCCCATCCAGCTAGAGTC
             || |||| ||
   3'      CTGAGATCGACCTACCCTTCG

3' Duplex: 2
   5' GCTTCCCATCCAGCTAGAGTC
                     ||
   3'                CTGAGATCGACCTACCCTTCG

3' Match Count: 4
   5' GCTTCCCATCCAGCTAGAGTC
                     || ||
   3'                CTGAGATCGACCTACCCTTCG

3' GC Duplex: 1
   5' GCTTCCCATCCAGCTAGAGTC
                      |
   3'                 CTGAGATCGACCTACCCTTCG
```

Identified Misalignments Within a Candidate Primer-Set are presented for further review by the PCR practitioner. Those oligos recognized to hybridize in excess of a certain threshold are automatically eliminated from consideration by the design software.

Design software is also used to target certain features in the sequence. A common problem for gene expression analysis is the carry-over of genomic DNA contamination through RNA isolation and purification. Reverse transcription converts this RNA into cDNA, but any residual gDNA may also provide a target for amplification, introduce false positives and otherwise compromise quantification. This problem can be mitigated through treatment with DNase prior to reverse transcription, and also through careful selection of the oligos during assay design. Assuming the target gene has multiple exons, a common strategy is to position the primer-set across a splice junction within the transcript, where an intron sequence had been excised. In doing so, it is important to recognize that primers spanning a short intron sequence may continue to be amplified. Additionally, some pseudogenes lack introns and will be efficiently amplified from gDNA, despite designing the assay across a splice junction in the native gene. For each target under investigation, an understanding of its genomic context and sequence homologs is fundamental to assay design.

This is a small sampling of the numerous considerations that inform the selection of oligo sequences. They collectively improve the performance of de novo assay design, and qPCR design software will traverse them automatically. With all the power of computation, many applications will still challenge automated design algorithms. It is important to become familiar with the function of each design rule for those difficult target sequences that require deviating from default parameters, because any one parameter can usually be disregarded to generate a functional assay still. Many complex genetic tests require custom oligo sequences selected manually, while continuing to benefit from computation for evaluation of their properties.

Section 4

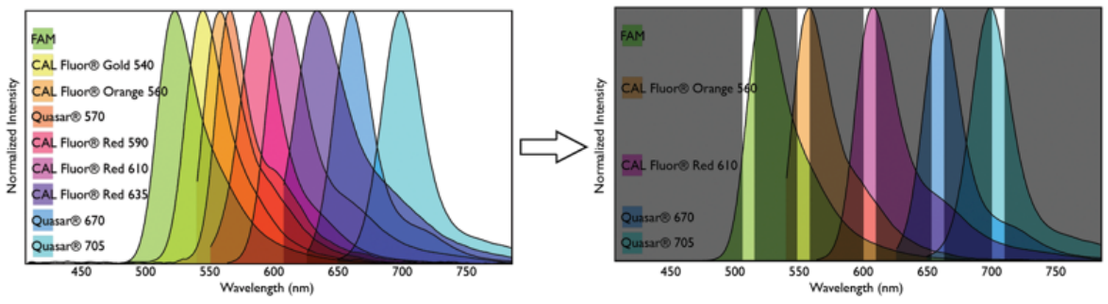
Advanced Considerations for Oligo Design

1. Multiplexing
2. Sequence Specificity
3. SNP Genotyping
4. Degenerate Bases

Multiplexing

Certain biological investigations are more sophisticated in scope, requiring significant development and validation. Many diagnostic tests and other commercial products combine multiple assays into the same reaction vessel to comprise a multiplexed set. The assays are amplified simultaneously but detected independently using different fluorescent reporters. It is therefore essential to select a dye combination with fluorescence spectra that are well resolved, while also remaining compatible with the real-time thermal cycler. These instruments vary considerably in their optics specifications, which typically range from 2 – 6 detection channels. One must consider the excitation source, whether laser, lamp or LED, and particularly the excitation/emission filters used for detection (Figure 4.17).

Figure 4.17 – Fluorescence Spectra & Instrument Optics



The Normalized Emission Spectra for a Series of Fluorophores provide a reference to choose candidates for multiplexing (left). Optimal reporters for a pentaplexed assay are identified by comparing spectra to the instrument's filter specifications (right).

Most real-time thermal cyclers are engineered for FAM/SYBR first and foremost, while longer wavelength fluorophores may be detected less sensitively. Many require dye calibration to accurately deconvolute the overlapping signals. Considering all these dependencies, it is helpful to consult the recommended dye combinations, whether that of the instrument or oligo manufacturer. For example, the instrument compatibility for the CAL Fluor and Quasar dyes can be found on the following webpage, accompanied by the spectra of other common reporters to overlay for comparison:

Spectral Overlay Chart

http://www.qpcrdesign.com/spectral-overlay

During oligo design, each additional assay in the multiplexed set increases competition for the same pool of reaction components, and exponentially increases the number of inter-oligo interactions. Again, software programs are best-suited to screen for primer-dimers among so many combinations, and several do include multiplexing modules for assay design. Multiplexed real-time PCR requires more time to implement than the same assays run independently, but ultimately serves to conserve sample material and control well-to-well variation. This approach achieves cost savings to an optimized assay that will be run repeatedly – ideal for applied markets with high-throughput processes.

Sequence Specificity

Certain applications have pronounced requirements for amplification specificity, to either tolerate or else distinguish between a collection of similar sequences. Examples include the selective amplification of a transcript among multiple splice variants, or to distinguish species according to their mitochondrial sequences. Within the genome are many repeated motifs, or whole families of genes that are very similar in sequence and thus difficult to distinguish. A test for infectious disease may require comparable amplification across a collection of isolates harboring different mutations, while also selecting against nearest neighbor

microorganisms. All of these applications require highly targeted assay design, with the oligos aligned to either conserved or else variable regions depending upon the intent. Automated software cannot anticipate all the biological considerations that inform assay design. Almost certainly these applications require manually positioning the oligos, even sacrificing criteria that might be considered gospel.

For such sophisticated assays one should consult public databases such as NCBI's RefSeq to obtain all available information about the target sequence and, once designed, then BLAST the primer sequences to gauge off-target amplification from alternative sites. Identification of the conserved or variable regions can be accomplished with a multiple sequence alignment to precede assay design. One such tool is Clustal, hosted by EMBL, and freely available over the web. An example assay requiring carefully-targeted oligos to ensure specificity of amplification is the following genomic sequence, a section of the Tceal3 gene in mouse:

AAGAAGTTCGCGGTGAAAACGAAGGCAAGCTGGAAAAGGAGGGAA
AGCCAGAAGATGAAGTAGAGCCTGAAGATGAAGAAAAGTCAGACGA
GGACGAGAAGCCAGACAAGAAAGCAAAGCCAGCACCACGCCAGG
GCAAGCCAGAGGAGGAGGCAAAGCCAGATGAGCAAGGGCAAGATG
AGGGGAAGCCCGAGAAGCAGGGAAAGTCTGACGGGGAGGGCAAG
CGCCAAGGGGAGAGCAAGCCCGATTCCCAGGCAAAGTCAGCCAGC
GAGGCGCGGGCTGCAGAAAAGCGCCCTGCTGAAG

This sequence shares homology to Tceal5 and Tceal6, representing other members in the gene family. Primers for this assay are designed to selectively amplify Tceal3 to the exclusion of the gene homologs:

Forward: 5'-GCCTGAAGATGAAGAAAAGTCAGAC-3'

Reverse: 5'-GCCCTTGCTCATCTGGCTTTG-3'

Figure 4.18 – Multiple Sequence Alignment of Gene Homologs

```
Tceal3      AAGAAGTTCGCGGTGAAAACGAAGGCAAGCTGGAAAAGGAGGGAAAGCCAGAAGATGAAG
Tceal6      .......C.....................................................
Tceal5                  .....T.....A....CA.....CA......G.G.......C....

Tceal3      TAGAG-CCTGAAGA-TGAAGAAAAGTCAGACGAGGACGAGAAGCCAGA--CAAGAAAGCA
Tceal6      .....-........-.............T.....A...........--..........
Tceal5      G.-..TA.A.....GG..G......-....T..--.-..C...T....TG.-..-.G.GG

Tceal3      AAGCCAGCACCACGCCAGGGCAAGCCAGAGGAGGAGGCAAAGCCAGAT--G-A-GCA-AG
Tceal6      ..............................................--.-.-...-G.
Tceal5      .......---.............T.....T.....G.GG.....G.GA.C.G...C..

Tceal3      -GGCAAGATGAGGGGAAGCCCGAGAAGCAGGGAAAGTCTGACGGGGAGGGCAAGCGCCAA
Tceal6      -.....................................................------
Tceal5      CA.A...G.....................................................

Tceal3      GGGGAGAGCAAGCCCGATTCCCAGGCAAAGTCAGCCAGCGAGGCGCGGGCTGCAGAAAAG
Tceal6      .....................................................------
Tceal5      .....................................................------
```

Multiple Sequence Alignment of Tceal3, Tceal5, and Tceal6 genes Signature bases that differ from the intended target are annotated with either A,C,G,T while ... represent base positions that are identical. Gaps are indicated with dashes. Binding sites for the actual primers selected for this assay are highlighted in gray.

Specificity for the intended target is improved by aligning the oligos to regions of mismatches in the gene homologs, particularly at the 3' terminus of each primer (Figure 4.18). This design strategy is known as allele-specific PCR, and serves to delay the onset of amplification from those sequences that are not fully complementary (Newton et al., 1989). Amplification from the gene homologs can be suppressed entirely with sufficient mismatches, although that outcome depends on reaction conditions and the prevalence of the related sequences within the background DNA. Specificity can also be improved by aligning the probe across the signature residues. This reduces the binding stability of the probe to mismatched sequences and diminishes the signal magnitude in response to off-target amplification, depending on the number of mismatches and the length of the probe.

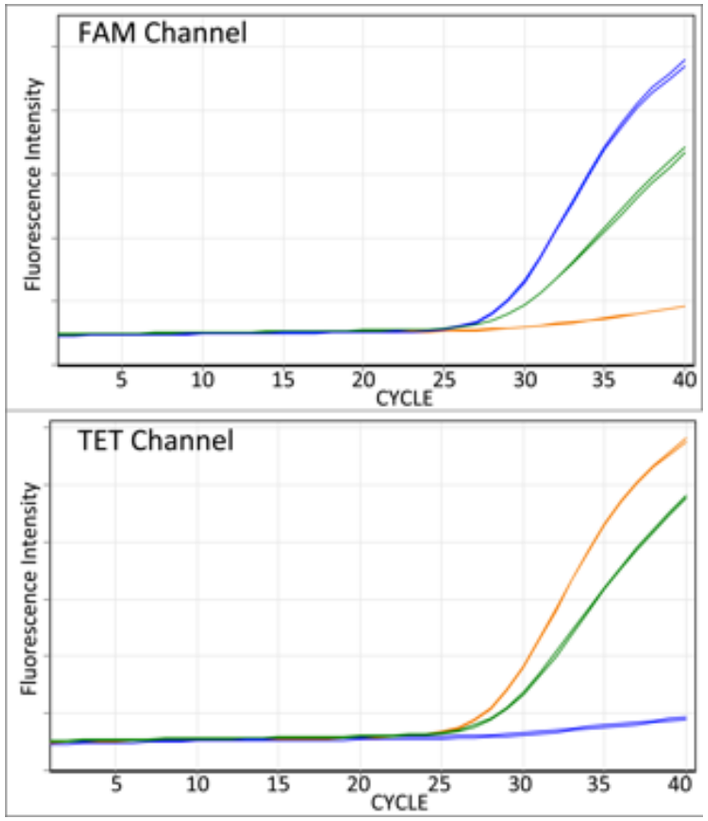
Despite the best attempts at design, some off-target amplification may still be detected with either allele-specific primers or allele-specific probes, or even the combination of both. These assay designs typically require empirical testing and optimization, particularly when interrogating a single base difference. A solitary mismatch can be very difficult to distinguish with 100% specificity, and yet single nucleotide polymorphisms (SNPs) are a very popular target for PCR, whether studying the genetic predisposition for disease among a population or tracking the phenotype of seed stocks in agriculture.

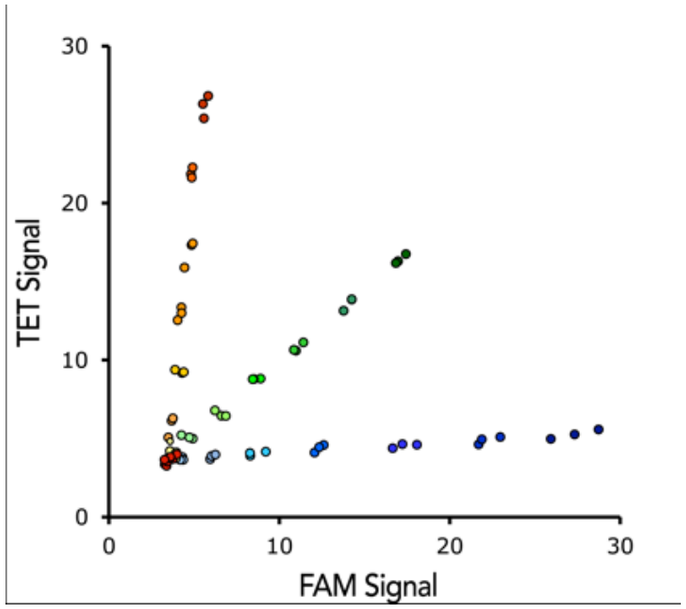
SNP Genotyping

SNPs are found in both coding and non-coding regions of the genome, and represent the most common form of genetic variation. Given their prevalence and utility, there exist many different techniques for genotyping including sequencing, microarrays, and high resolution melt curves. Probe-based genotyping uses the PCR process to amplify the flanking sequence, in conjunction with hydrolysis probes to bind the SNP and signal the genotype. Two probes are labeled with different reporter dyes that are each fully complementary to one of the two alleles. By competing to bind the same target sequence, the allele-specific probes gain specificity over either probe acting individually, and the fluorescent signal from the matching probe predominates.

In practice, some cross-reactivity can be expected where one or both probes bind their mismatched allele, but with a reduced signal response compared to the fully-complementary allele. For this reason the genotype determination is not typically assigned according to a yes/no amplification event, but rather a scatter-plot of the end-point fluorescence intensity of the two dyes. Homozygous samples tend to fall along each axis while the heterozygous genotype clusters toward the middle of the plot.

Figure 4.19 – Probe-Based Genotyping





Allele-Specific Probes labeled with FAM and TET produce a different amplification response (left) from samples representing homozygous wild-type in blue, homozygous mutant in orange, and heterozygous in green. A scatter-plot is used to make genotype determinations, in this case presenting a dilution series of the samples (right).

Probes for SNP genotyping require a careful compromise between longer sequences that promote binding stability and therefore increased signal response, and shorter sequences that promote mismatch discrimination and therefore minimize cross-hybridization. In practice, this balance is not easily achieved while using traditional probes 20 – 30 bases in length. Genotyping benefits from additional modifications to fortify the probes with increased binding stability. Certain chemistries are used to shorten the oligo sequence while maintaining a proper TM, including the MGB moiety or the propynyl bases within BHQplus probes. These modified probe-types are more commonly used for SNP genotyping.

Degenerate bases

Oligos are occasionally designed to tolerate a known variation in the sequence, or when there is uncertainty as to the correct sequence. This situation can be addressed by incorporating one or more degenerate positions in the oligonucleotide. The

degenerate residue, or “wobble,” does not represent a discreet nucleotide but rather one of several different nucleotides at that position. They are manufactured by combining two or more base phosphoramidites at equimolar ratios before coupling, producing multiple sub-sequences from a single oligo synthesis. Wobbles are designated by the standard IUPAC code for mixed bases (Table 4.2).

Table 4.2 – IUPAC Base Code.

IUB Base Code Guide

Standard Bases

Code	Base
A	Adenosine
C	Cytidine
G	Guanosine
T	Thymidine

Mixed Bases (Wobble)

Code	Base
B	C, G, or T
D	A, G, or T
H	A, C, or T
V	A, C, or G
R	A or G
Y	C or T
K	G or T
M	A or C
S	G or C
W	A or T
N	Any Base

This design strategy is commonly practiced for pathogen detection, for example to tolerate multiple different isolates while detecting a virus with a high mutation rate. However, it is important to remember that each additional wobble exponentially increases the number of sub-sequences synthesized in unison, thereby decreasing the likelihood that any individual sequence has the desired specificity. Only one species is usually present in a biological sample and so amplification performance may be compromised with some portion of the oligos not fully complementary. It is advisable to be conservative and introduce as few degeneracies as possible.

A similar approach is to introduce a “universal” base like inosine, which base-pairs to either A, C, G, or T (Loakes, 2001). It is important to remember that these non-standard bases are not truly universal—they may show preference for some nucleotides over others. Despite their limitations, both design strategies are quite useful when the primer or probe must tolerate a known mutation and the sequence space is otherwise limited.

Section 5

Databases of Pre-Designed Oligos

Many databases of pre-designed assays are available through the internet, and may reduce the risk of new assay development if the desired target is represented within. They are usually oriented toward gene expression analysis and SNP genotyping within model organisms, while assays for other applications and species are sparsely represented. Some resources are simply a repository of computer-generated primers without performance verification, while others contain wet-lab validated oligos with accompanying quality data (Lefever et al., 2009; Wang and Seed, 2003; Wang et al., 2012). Despite the lack of a single unified database for all organisms there are useful specialized databases available through the web such as PrimerBank and RTprimerDB.

Many validated qPCR assays are available from commercial sources, usually in the form of a kit. The product documentation may not reveal the oligo sequence and yet those specifications represent an essential reporting requirement of the MIQE standards. While controversial, the manufacturer's catalog number should be referenced in lieu of sequence information during publication, or else the assay ID from within a commercial database of pre-designed assays that are similarly kept confidential.

Experimentally validated qPCR assays can be identified through careful research of the literature, but reliance on published data highlights the importance that authors and journals adhere to the MIQE guidelines so that other researchers

might utilize or replicate their qPCR assays. Previously published qPCR assays are a good reference, but before re-synthesizing the oligos it is important to critically evaluate the rigor of the author's research, the quality of their assay design, and any proprietary chemical modifications that influence assay performance.

While pre-designed assays may expedite development, all the same requirements for experimental validation remain as relevant as when testing a de novo design. Seemingly innocuous changes can have a profound impact on amplification, like a different fluorescent label for the probe or a different polymerase formulation. Given all the subtle variations between each set-up, functional testing with the proper positive and negative controls is essential whether the assay was purchased as a kit from a vendor, whether repeating previously published work, or else developing a new assay with no precedent.

Section 6

Conclusion

Genetic methods are easier to implement than ever before due to the efficiency of oligo manufacture and ubiquity of pre-designed assays. While qPCR has matured into an established technique, the principles of oligo synthesis and design should not be neglected. To troubleshoot an unanticipated result requires appreciation of the underlying principles, whether the reactive precursors in chemical synthesis, or the signaling mechanism of a hydrolysis probe. Scientists can only hope to recapitulate previously published works through comprehensive reporting as dictated in the MIQE guidelines: sequence, modifications, target location, and manufacture. In combination with sophisticated real-time instrumentation and thermostable enzymes, it is the oligos that accomplish target recognition and trigger the chain reaction. Residing at the interface of chemistry and biology, oligos are the critical components of qPCR.

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Chapter 6

qPCR Instrumentation

Application of the MIQE Guidelines to the Selection of qPCR Instrumentation

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Summary

The discovery of Real-Time or quantitative polymerase chain reaction (qPCR) in the 1990s took PCR to a new level: the ability to monitor the DNA amplification process not after the fact, but as it was happening. Such monitoring requires specialized instrumentation capable of conducting the enzymatic process and collecting real-time data simultaneously in the same reaction vessel. For years, these experiments were plagued by a lack of consensus in the reporting and interpretation of the qPCR data. Today, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines have set forth a baseline that increases transparency, promotes global consistency and helps to ensure the integrity of the scientific literature (Bustin, 2009); selecting the right instrumentation is key for ensuring accurate, reproducible results.

Section 1

History of instrumentation

In 1983, while on a drive through Mendocino County in California, Kary Mullis — a researcher with Cetus Corp. — was deep in thought about a problem that had been plaguing him at work: how to find a way to concentrate a single DNA locus in the presence of millions of similar DNA loci. The answer was polymerase chain reaction (PCR).

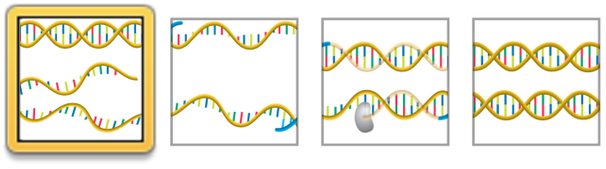
By combining primers and DNA polymerase with thermal cycling, PCR doesn't replicate the entire strand of DNA, but a specific target sequence. The target DNA multiplies in a chain reaction that provides exponential amplification, generating billions or trillions of copies in a targeted timeframe. (Karymullis.com; Saiki, 1988), This technology has been adapted for numerous molecular biology applications (VanGuilder, 2008), and won Mullis the Nobel Prize for Chemistry in 1993. (nobelprize.org)

However, the process was dependent on end-point analysis; researchers wanted the ability to measure during the log phase of the reaction. Just a decade later, Russell Higuchi and colleagues at Roche Molecular Systems and Chiron demonstrated the discovery of Real-Time or quantitative PCR (qPCR) by connecting a thermal cycler to a spectrofluorometer. (Higuchi, 1992; Higuchi, 1993), With qPCR, minimal amounts of DNA can be replicated rapidly and detected easily; amplicons are detected while the reactions are in progress

through fluorescent signalling. In contrast to traditional PCR, qPCR is able to ascertain up- or down-regulation of a particular DNA sequence, or how much of a specific DNA sequence is present in a sample, rather than simply identify its presence or absence.

The PCR Principle. PCR is a cyclic DNA amplification process. Each cycle involves three steps, which are described in detail above.

Denaturation of template by heat



As PCR amplification and detection takes place in the same tube, qPCR no longer requires post-amplification analysis steps that had been traditionally associated with PCR, such as purification and dilution of PCR products for downstream work, or confirmation of assay specificity by gel electrophoresis, though such steps can still be taken if desired. This real-time measurement reduces opportunities for contamination and variability in the results.

With qPCR, the speed, sensitivity, and reproducibility of homogeneous assays and the limited risk of carryover contamination have made it a standard for nucleic acid quantification. (Mackay, 2004) Today, qPCR is the gold standard methodology used for determination of DNA copy numbers, quantitating transcription, and other applications. (Mann, 2006)

Yet prior to the introduction of the MIQE guidelines (Bustin, 2009), there was a lack of consensus on the use of standardized reagents, protocols, analysis methods, and reporting formats. (Bustin, 2004)

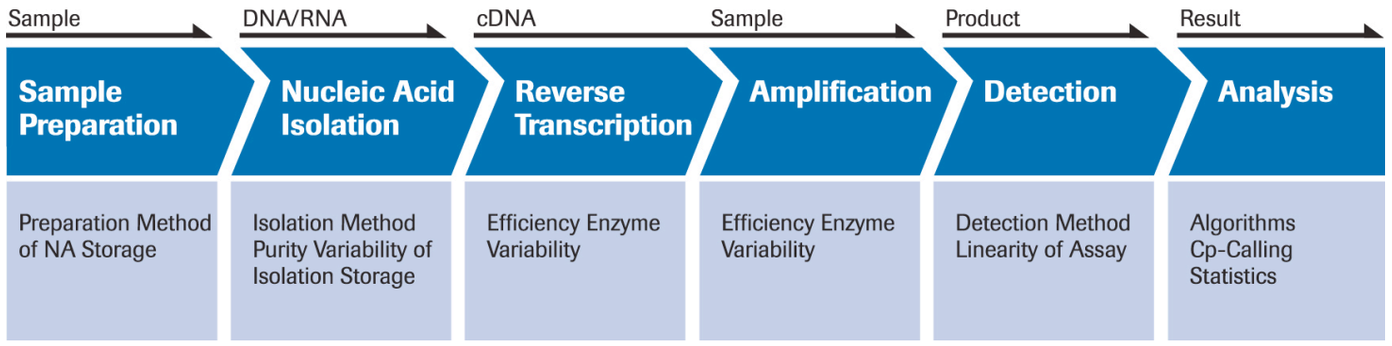
Section 2

Methodology

Real-Time or qPCR occurs in a computer-controlled imaging thermal cycler. These thermal cyclers consist of the following components: (Morrison, 2006)

- Software that controls operation, collection and analysis of generated data
- A thermal cycler
- An excitation/emission system capable of exciting fluorophores at optimal wavelengths and then capturing the Stokes shift emission wavelength
- An imaging system to capture the fluorescence generated by the process

Criteria to be considered when planning a gene expression assay.



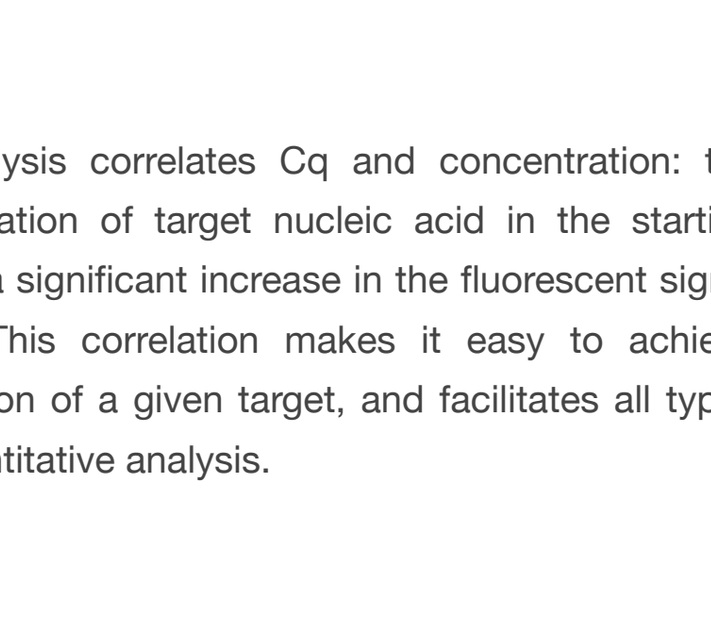
With every PCR cycle, the product amount increases and becomes observable when the fluorescence intensity exceeds background levels. Fluorescence is a type of luminescence generated when a substance absorbs light energy at a short, high-energy wavelength, and then emits light energy at a longer, low-energy wavelength. Only a fraction of a second passes between absorption and emission. The cycle at which the fluorescence becomes visible is called the crossing point (Cp) or quantification cycle (Cq). The PCR quantification is performed at this stage, when the rate of amplification should be at its maximum, ideally with the amount of product approximately doubling with every cycle.

qPCR instrumentation allows researchers to follow the amplification of DNA molecules and the accumulation of PCR products in real-time — indirectly, by way of fluorescence measurements. Detailed analysis of the sigmoidal curve profile reveals a consistent pattern: an early background phase; a period of exponential growth (log linear phase); and a period of reduced activity, known as the plateau phase.

Quantification – the Q of qPCR – is relatively straightforward. The more copies of the target at the start of the assay, the fewer amplification cycles that are needed to generate enough amplicons for fluorescence to reach detectable thresholds. (Bustin, 2005)

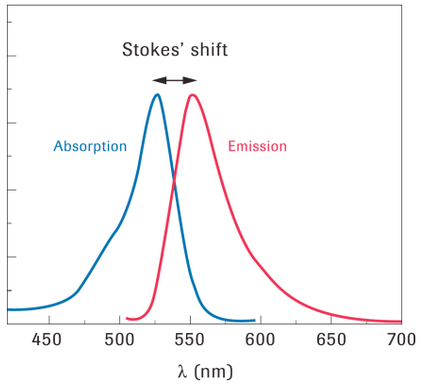
In qPCR, data analysis correlates Cq and concentration: the higher the concentration of target nucleic acid in the starting material, the earlier a significant increase in the fluorescent signal will be observed. This correlation makes it easy to achieve sensitive quantification of a given target, and facilitates all types of qPCR-based quantitative analysis.

Amplification curves in Real-Time PCR; correlation between Cp and concentration.



In the qPCR process, a fluorescent probe is a sensitive and selective tool used to detect a key biomolecular target. Fluorophores are molecules that display fluorescence; each has characteristic absorbance (or excitation) and emission spectrums, and will most efficiently absorb and emit energy at set wavelengths, known as peak absorbance and peak emission, respectively.

Energy is supplied by an excitation source — LED, Xenon or halogen light — creating an excited state that lasts for just nanoseconds and is detected by an excitation filter designed to selectively identify light from the excitation source. A second filter captures the peak emission. The Stokes shift is the difference between the peak of absorption and emission spectra, as seen in figure below.



Stokes shift

With known peak absorbance and peak emission wavelengths, it's easy to identify the Stokes shift for each fluorophore. The situation becomes significantly more complicated when multiplexing. Multiplex PCR is a rapid and convenient screening assay that uses multiple primer sets simultaneously to identify multiple targets. Yet it requires strategic planning to optimize reaction conditions, ensuring that optical crosstalk between the different fluorophores do not cause false positive Cq calls.

Section 3

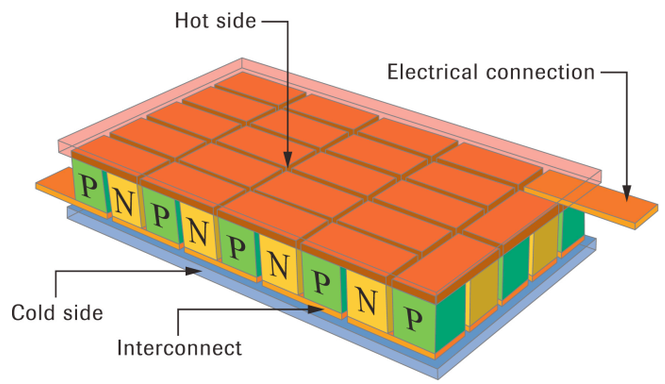
Thermocycling

The thermal cycler is responsible for automating the temperature cycling and control. A qPCR assay's specificity and yield are directly related to the ability of the thermal cycler to rapidly and accurately arrive at target temperatures (Wittwer, 2014), and the ability to maintain temperature stability and homogeneity across wells. Temperature irregularities across wells can create inconsistencies that affect fluorescence signals and primer annealing. Some applications — such as high-resolution melting — must be able to distinguish extremely small temperature differences, variations as small as 0.2° C. Most modern devices report thermal uniformity in the range of ±0.2°C to ±0.5°C.

Heating methods

Much of today's qPCR instrumentation builds upon Peltier-based heater block systems. A Peltier element has two sides; when DC current is applied, the block transfers heat from one side to another, resulting in a "hot" side and a "cool" side. The direction in which the current flows across a junction determines whether heat is liberated or absorbed. (Pollock, 1993) They are compact, solid-state devices with no moving parts, and do not require maintenance. Peltier elements are a common component in many scientific devices for their efficiency and ability to control temperatures to within a fraction of a degree. Most manufacturers of Peltier-based blocks use 4-6 Peltier elements; the block is designed to dissipate the heat for uniformity, minimizing hotspots.

Peltier-based block - details



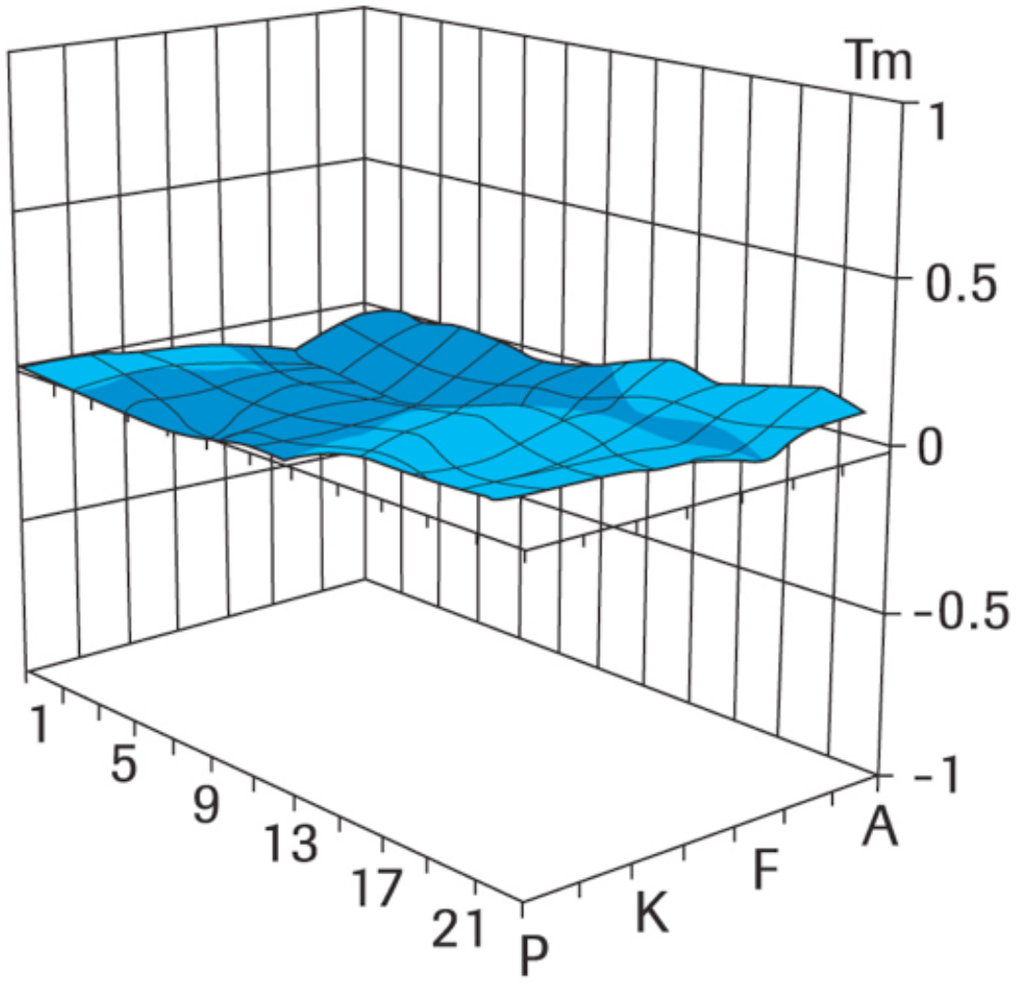
Conventional Peltier systems are relatively slow; the solid block maintains a great deal of thermal inertia. This, combined with the extra insulation provided by the plastic coating of the reaction vessels, makes it difficult to heat or cool quickly. As a result, manufacturers have further improved upon the Peltier-based system through the addition of resistive or convective technologies to improve control, performance and thermal ramp rates across the block. These technologies include:

- Precisely electroformed, hermetically sealed silver blocks filled with a thermally conductive fluid; opposing agitators rapidly circulate the fluid, quickly transferring heat from the single Peltier device
- The use of a heat-equalizing layer beneath the Peltier element that ensures optimal heat distribution and well-to-well homogeneity

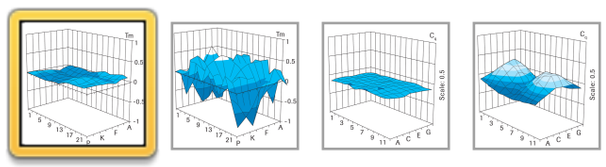
Other manufacturers use non-Peltier systems to achieve faster ramp rates. These techniques include:

- Glass capillary systems heated and cooled by high-speed turbulent air
- Centrifugal air-heated plastic tubing, which offers excellent uniformity and the ability to cool more quickly
- A highly thermally conductive ceramic heating plate coupled with a high-efficiency fan for heat distribution
- Microcontroller-driven, silicon-based resistive heating elements covered by a glass substrate

By minimizing the amount of metal involved in the heating element, heat is transferred more efficiently, making it easier to adjust the temperature up or down. However, this increased uniformity restricts the ability to have a temperature gradient, but as gradients are only used in assay optimization, many laboratories prefer the benefits of uniformity for their day-to-day operations.



Influence of the Therma-Base layer on temperature homogeneity across a 384-well plate. LightCycler® 480 Real-Time PCR System including Therma-Base



Section 5

Assay performance

Assay performance is essential for the critical evaluation of data included in each submitted manuscript. Performance is judged primarily on four characteristics: PCR efficiency, linear dynamic range, LOD, and precision.

Robust assays are correlated with high PCR efficiency. (Bustin, 2009) Efficiency is established by means of calibration curves. This calibration is determined from the slope of the log-linear portion of the calibration curve, and offers a reproducible indication of efficiency, sensitivity and robustness of the assay.

The **Linear Dynamic Range** — the highest to lowest quantifiable copy number — that includes the interval for the target nucleic acids being quantified.

The **LOD**, or limit of detection, is the lowest concentration at which 95% of positive samples can be detected; conversely, no more than 5% of failed reactions should occur.

In qPCR, **precision** typically varies with concentration, although temperature differences and other variations can occur.

The performance of qPCR assays can be affected by changes in primer and probe concentration, cycling conditions, and buffer concentration. Primer and probe sequences should be published to ensure consistent and reproducible results per the MIQE guidelines; commercial primer and probe sequences are readily available. (PrimerDesign) A well-designed assay will show few or no test-to-test variations in Cq and fluorescent signal intensity.

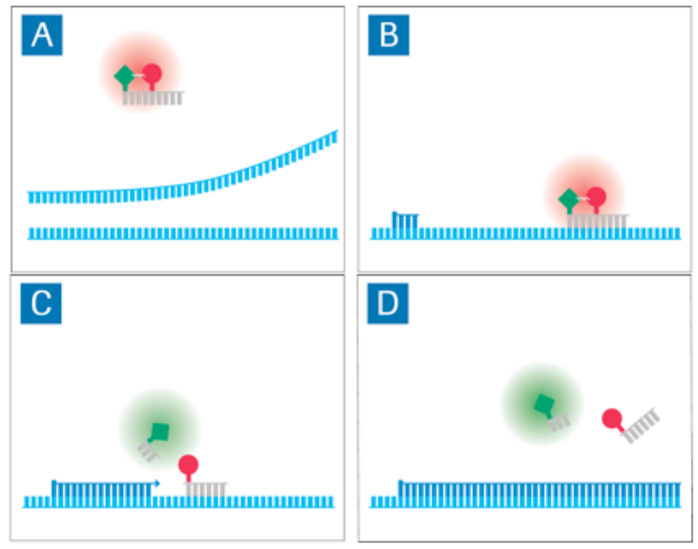
Primers and probes

All qPCR systems use fluorescent dyes for detection. The most commonly used assay formats are as follows:

Hydrolysis probes

(also referred to as **TaqMan** probes) are highly specific, significantly increasing the specificity of fluorescence detection and permitting quantitative measurement of the accumulated concentration. The reporter dye is released by hydrolysis during PCR amplification.

Hydrolysis probes

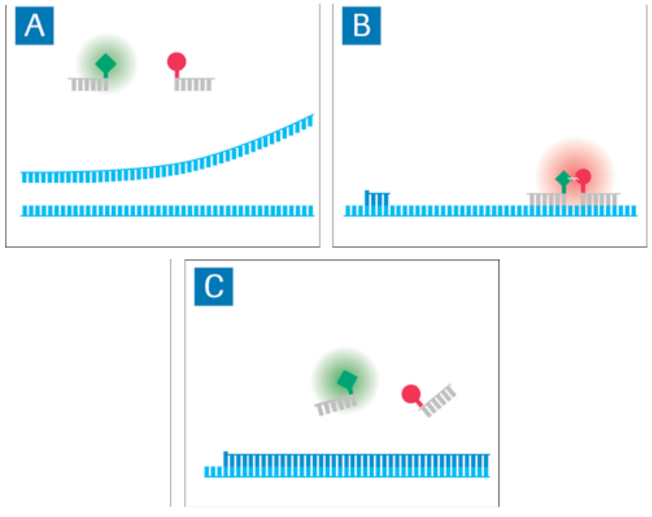


Panel A-D show the behaviour of hydrolysis probes during PCR. The probe carries two fluorescent dyes in close proximity, one of which (quencher) quenches the fluorescence from the other (reporter) as long as the probe is intact.

Dual-hybridization probes

(also known as **HybProbes** or **FRET** probes — fluorescence resonance energy transfer) rely on donor fluorophores transferring energy to acceptor fluorophores. The acceptor fluorophore emits a detectable light of a longer wavelength; donor excitation light alone cannot excite the acceptor, and the energy transfer cannot take place unless the target DNA is present.

Dual-hydridization probes

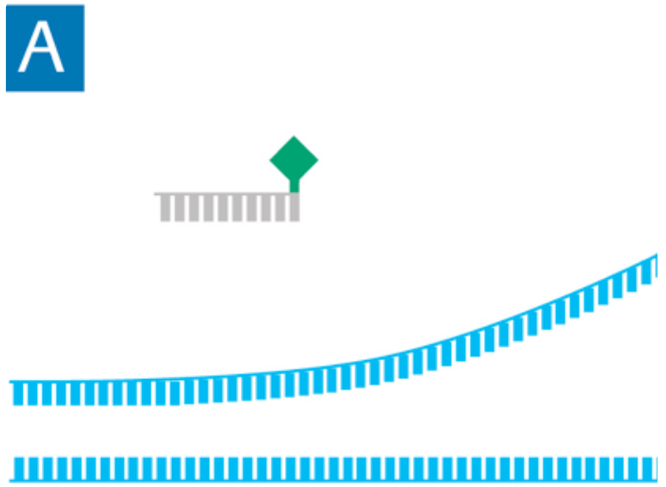


Panels A-C show the behaviour of HybProbe probes during different states of PCR. In the example shown, the donor dye probe has a fluorescein label at 3' end, and the acceptor prove has a LightCycler® Red label at is 5' end (note that this setup might also be reversed, i.e., donor dye at the 5' and acceptor dye at the 3' end).

SimpleProbe

is a special type of hybridization probe. Instead of two probes per assay, like HybProbes, only a single probe is needed. This probe carries a covalently attached dye that hybridizes to a target; once hybridized, it emits greater fluorescence.

Simple probe



During the denaturation phase no hybridization takes place, thus only a low fluorescence background is detected at 530 nm (panel A).

SYBR Green dye binds preferentially to double-stranded amplicons during PCR and is used in gene expression analysis and DNA quantification, generating fluorescence signal when subjected to light of an appropriate wavelength.

Scorpions Probes are a single-stranded, bi-labeled fluorescent probe/primer hybrid in a hairpin loop configuration. The probe is physically coupled to the primer. Scorpions are efficient and have fast reaction times and an exceptional signal-to-noise ratio. The polymerase synthesizes the complementary strand of the target sequence; the loop unfolds and hybridizes to the newly synthesized target. Now that the reporter is no longer in proximity to the quencher, fluorescence emission takes place.

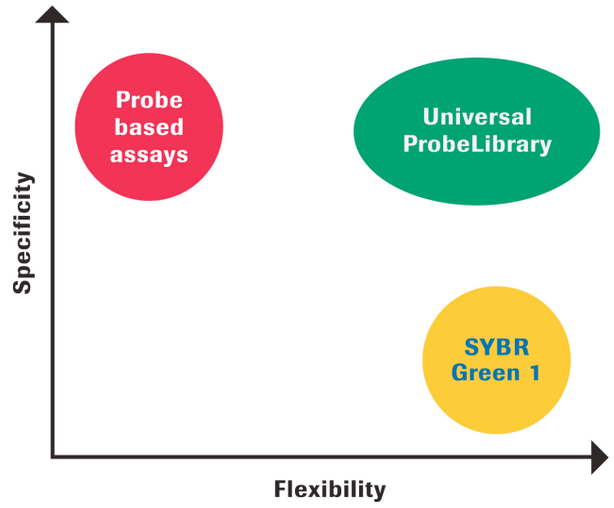
Molecular Beacons are able to report the presence of a specific nucleic acid in a homogeneous solution. They are single-stranded probes in a hairpin loop configuration. They bind to the amplified target, and the resulting fluorescence is dependent upon the amount of product. Molecular beacons are increasingly used in multiplexing.

Locked Nucleic Acids (LNA) oligonucleotides are thermally stable, high-affinity RNA analogues. Varying the LNA content of the oligonucleotide can optimize specificity and sensitivity. It normalizes results across a series of short sequences with varying GC content.

ROX Reference Dye was introduced in the 1990s as a passive reference dye, and used to normalize for non-PCR fluorescence signal variation on some real-time thermal cyclers. ROX is an inert dye that maintains stable fluorescence throughout the qPCR reaction; it provides a reference point to which reporter dye signals can be normalized during data analysis. It offers a stable baseline in multiplex qPCR reactions, and normalizes well-to-well differences that can result from pipetting errors. Applying ROX normalization decreases the standard deviation.

Significant improvements in design and manufacturing of newer generations of systems have obsoleted the need for ROX as a reference dye.

UPL assays represent a unique combination of specificity and flexibility.



Instrumentation Features and Selection

Instrumentation varies across manufacturers. Selecting the correct system requires an understanding of each device's mode of application, product features, required functionality, and the ability to upgrade and grow the system as your needs change — such as the ability to switch out the blocks, from 96 to 384.

Sensitivity: There are two key forms of sensitivity when discussing PCR: analytical sensitivity indicates the minimum number of copies that can be accurately measured with a given assay (also known as limit of detection or LOD), and clinical sensitivity, which identifies the percentage of individuals identified as positive for a given condition.

Reproducibility: The same samples should return the same results in different runs, or when tested by different labs. While more robust assays will provide more reproducible results, it is important to look for devices that eliminate well-to-well variation in temperature and light source.

Xenon bulbs emit a continuous spectrum, offering high luminance and color temperature ranging from UV to infrared. Light emitting diodes (LED) are opto-semiconductors that offer bright light, a long lifespan, and cool operation. Halogen

and halogen-blend lamps produce a bright, continuous spectrum of light, from infrared to deep ultraviolet.

Accuracy: High-performance optical components ensure uniform data collection across a multiwell plate, regardless of sample position. **Charge coupled devices (CCD)** are small, integrated circuits etched onto a silicon surface, capable of detecting the surface charge generated by photons. CCDs are multichannel detectors and have the highest light sensitivity, with low noise and dark current, giving them a large dynamic range, yet they aren't always the best for every application. CCDs are so sensitive that they are subject to vignetting, wherein the angle of the light hitting the sensor can cause a reduction in brightness in the periphery of the image.

Photomultiplier tubes (PMT) are larger vacuum phototubes with excellent detection within the ultraviolet spectrum. PMTs can generate signals faster than CCDs, and do not have the same vignetting issues that are seen with CCDs.

Reliability: Research and development and the quality control that go into a system can have an impact on functionality, use and device usability.

Ease of Use: Is the device understandable? Easy to use? Does it guide you through operations to give usable, understandable data for users of all experience levels? Most qPCR software supports straightforward Cq calculation of the recorded fluorescence, but this data often requires additional analysis — such as averaging, normalization and statistical testing — to achieve meaningful results. (Wong, 2005)

Ramp Rates and Throughput: Faster cycle times can be achieved with the aid of specialized primers, and by combining annealing and extension into a single step. This process can reduce cycle times by more than half.

High-throughput screening applications are increasingly critical to pharmacogenomics research. Select instruments are now capable of analysing hundreds of genes within thousands of samples at a time, accurately delivering tens of thousands of data points in a single day.

Flexibility: Some devices offer modular designs with the ability to swap thermal block cyclers (96- and 384-well formats, for example), custom pairing of optical filters, and choice between clear or white multiwell plates.

Some instruments are best suited to ready-to-use reagents that eliminate the guesswork and are designed to support each of the main qPCR applications for both standard and fast-ramping protocols to maximize signal quality and reproducibility. Other devices offer the flexibility for using customized filters that have been calibrated onsite.

Applications: Select qPCR platforms have application flexibility, are capable of gene expression, gene detection, genotyping, and gene scanning (HRM).

There has been considerable growth in the popularity of HRM analysis in recent years. Melt curves are used to analyse the purity of reaction products following qPCR. Identifying melt profile differences requires specialized software and dyes to highlight small variations such as single-nucleotide polymorphisms (SNPs).

There are four classes of SNP within the human genome. (Venter, 2001)

The four classes of SNP within the human genome

SNP Class	Base Change	Frequency in the Human Genome
I	C>T, T>C, G>A, A>G	64%
II	C>A, A>C, G>T, T>G	20%
III	C>G, G>C	9%
IV	A>T, T>A	7%

Class IV SNPs are rarest and most difficult to identify; the transposition of the A and T bases result in the smallest melt curve temperature shift, requiring an instrument with extreme precision and thermal control.

Multiplexing: Multiplexing — allowing two or more targets to be amplified simultaneously by using multiple primer pairs in the same reaction tube to produce amplicons specific to different DNA sequences — expands the throughput of qPCR. Typically, multiplex PCR detects a target gene in one channel and a control gene in another.

Overview of popular fluorophores used in real-time PCR systems.

However the presence of multiple primers can lead to preferential amplification of one target sequence, or crosstalk between channels, wherein one target's amplification may inhibit that of the second. Detection and differentiation become critical, making it particularly important to establish and test comprehensive primer design parameters.

Section 8

Conclusion

Today, there are many instrumentation platforms available in the marketplace, from simple single-channel instruments to highly sophisticated high-throughput systems capable of multiplexing multiple targets. Delivering quality, reproducible results requires the selection of the correct instrumentation for the job.

Section 9

Literature

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Chapter 7

qPCR consumables

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Section 1

Introduction

Quantitative PCR (qPCR), with its numerous modifications, remains a mainstay of sensitive and reliable detection and quantification of nucleic acids in the life sciences as well as in the fields of medicine, diagnostics, forensics and agriculture. Its practical simplicity, along with its speed and sensitivity, have made it an indispensable tool for many applications, including microbial quantification, gene expression profiling, identification of transgenes in animal models and foods, risk assessment of cancer recurrence and applications for forensic use (Bernard *et al*., 2002; Mackay *et al*., 2002; Mackay, 2004; Bustin *et a*l., 2006).

However, despite its conceptual simplicity, qPCR is not a trivial technique, and accurate collection, analysis and interpretation of qPCR data may pose challenges. Variable pre-assay conditions, poor assay design and improper data analysis have been shown to contribute to inaccurate results and erroneous conclusions in qPCR experiments (Johnson et al., 2014). Technical or operational deficiencies may affect the quality, performance and validity of qPCR experiments, and these factors have been delineated by MIQE guidelines in order to prevent publication of inconsistent, inaccurate or misleading data (Johnson et al., 2014).

MIQE guidelines identify key steps in the qPCR experimental workflow which may affect its performance; these include sample/template, assay optimization/ validation, reverse transcription/assay and data analysis. So far, however, the importance of disposable plastic consumables and their overall impact on the

experimental workflow of qPCR has been taken into consideration only marginally, if at all (qPCR Webinar at LabRoots: https://www.labroots.com/webinar/bread-butter-method-genomics-improve-q-pcr). This is unfortunate, since laboratory plastics, such as tubes, tips and plates, are integral parts of any laboratory and qPCR workflow. Contrary to common belief, plastics may have a direct and dramatic impact on experimental outcome and data validity. In this chapter, the importance of laboratory consumables for successful qPCR/PCR will be discussed, as well as their influence on critical aspects within the workflow: pipetting precision/reproducibility, sample integrity/quality and assay sensitivity/reproducibility. Practical recommendations on how to estimate and significantly reduce the risk of affecting qPCR experiments through consumables are also summarized.

The influence of pipette tips on pipetting precision and reproducibility

Within the scientific community, an increasing number of qPCR experiments published cannot be fully reproduced by other groups. Among many factors, insufficient precision and reproducibility of the pipetting system may be an important reason. Whereas incorrect pipette handling or pipetting techniques are considered obvious sources of error, the pipette tips themselves are generally not taken into consideration. Flawed, low quality pipette tips may lead to incorrect or inconsistent pipetting volumes and thus to faulty or non-reproducible data. This is often discovered once experiments are repeated by other groups using different consumables (Liquid handling Webinar at LabRoots: https://www.labroots.com/webinar/improve-reproducibility-liquid-handling-crucial-factor-exemplarily-shown-ngs-elisa-qpcr,

Tip of the Iceberg Webinar at LabRoots: https://www.labroots.com/webinar/tip-iceberg-pipette-tips-influence-assay-results).

The influence of tips on the performance of the pipetting system

In the internal study performed by Eppendorf (Art *et al.*, 2016), tips from 15 different manufacturers were assessed with respect to precision and reproducibility. Surprisingly, the results indicated that a given pipetting system performs within specifications only when using tips recommended by the pipette

manufacturer. With the majority of tips tested, the reproducibility and precision of the assessed pipette did not meet its specifications.

As shown in Figure 1, the specified systematic error was exceeded in 5 cases of 1 µL tips and in 4 cases of 1,000 µL tips. Moreover, three of the 1,000 µL tips even exceeded the permissible systematic error stated by ISO 8655-1:2002, which allows wider tolerances than the manufacturer's specifications. In contrast, the random error was noticeably increased but remained within permissible tolerances. If all calibration results were combined, a total number of 8 of the 15 tip manufacturers failed to meet specifications.

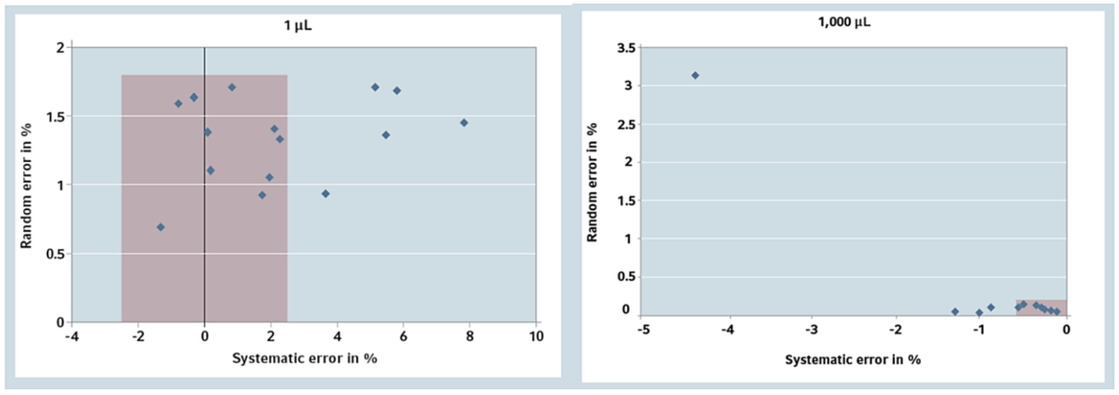

Figure 1. Calibration results using 1 µl and 1,000 µL tips from different manufacturers. The coloured area shows the maximum permissible error stated by the manufacturer for the pipetting system (Eppendorf Xplorer® pipette). All data points within the coloured areas are within the specifications. Source: Art *et al.*, 2016

These findings indicate that pipette tips may strongly influence pipetting precision and reproducibility and that they may be regarded as a source of major variation during sample preparation and even more so during qPCR assay setup. Furthermore, pipette users should be aware of the fact that when using tips not validated by the pipette manufacturer, the manufacturer's declaration or certificate of conformity does not apply. ISO 8655-21:2002 clearly states that the pipette and the tip constitute a system, and it recommends using the tips supplied by the pipette manufacturer. In case this is not possible, ISO 8655-21:2002 recommends that the user first perform a conformity test with tips from the pipette manufacturer. Once the pipette passes the test, a second calibration with tips not supplied by the pipette manufacturer must be carried out.

Tip design and its influence on pipetting results

Pipettes in general are calibrated to a certain air cushion size and filling height of liquid within the tip. Longer, bigger or slimmer tips lead to an increased total size of air cushion, which influences the filling level (Lochner et al., 1996). If the dead air volume increases, the pipetting volume decreases. Additionally, an increased filling height (slim and long tip) results in increased hydrostatic pressure

which must be compensated for and which also results in a decreased volume and higher systematic error (Salje, 2016) (Figure 2).

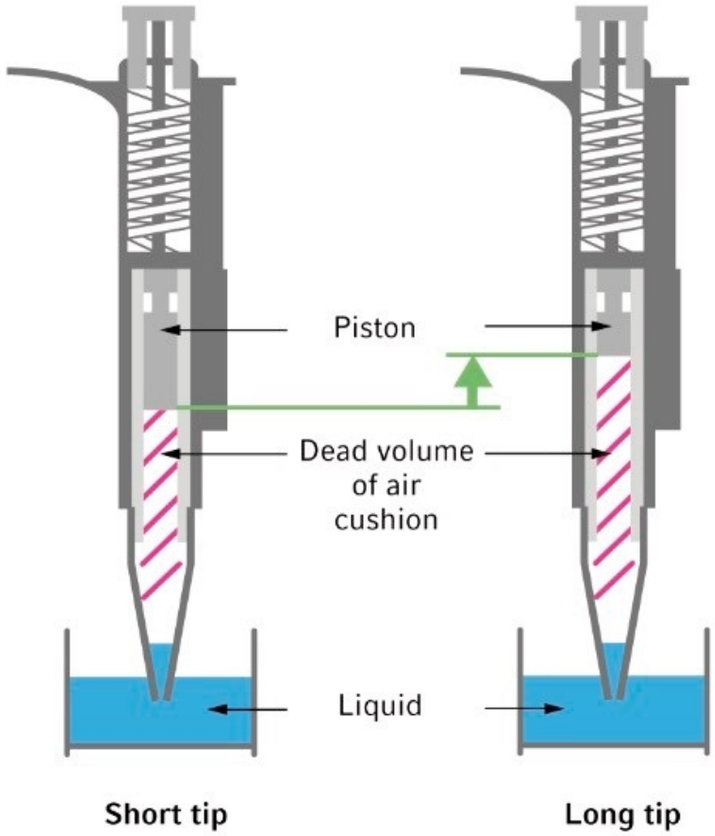

Figure 2. Influence of tip design on the dead volume of the pipetting system. Longer, bigger or slimmer tips lead to an increased total air cushion size and a different filling level.

The findings from the Eppendorf study (Art *et al*., 2016) indicate that these shape/size-related factors may play an important role regarding larger tips/volumes and negatively affect pipetting specifications in this volume range (Figure 1, 1,000 µL tips). This may affect various sample/buffer preparation steps in the qPCR workflow, which is contrary to the widely held belief that larger pipetting errors are limited to smaller tips and pipetting volumes.

Quality of tip orifice

The zone where the liquid leaves the tip during dispensing and where the drop cut-off occurs is very important for accuracy. Any imperfection of geometry or shape in this area, for example through production failures, may lead to sample retention, impair pipetting precision and sometimes make it completely impossible to dispense small volumes (small sample drops may remain attached to the tip). This effect may be of particular significance as it decreases the reproducibility and performance of the qPCR assay when pipetting very small volumes (total assay volume < 5 mL) (Nolan et al., 2013).

A poor orifice will typically not be perfectly round and/or it will have walls of varying thickness (Figure 3, manufacturer H). In such a tip, liquid drops may become deflected to the outside of the thinner part of the wall. Furthermore, "molding flashes" (Figure

3, manufacturers H, E and F) may lead to sample retention on the outer surface of the tip.

High quality tips should look very different: they must have a precisely defined, symmetrical wall thickness and a smooth surface structure to ensure a correct drop cut-off (Figure 3, Eppendorf tip). Importantly, these parameters must also be maintained at very low production tolerances, and throughout all tip sizes and production lots, in order to assure high inter- and intra-experimental reproducibility.

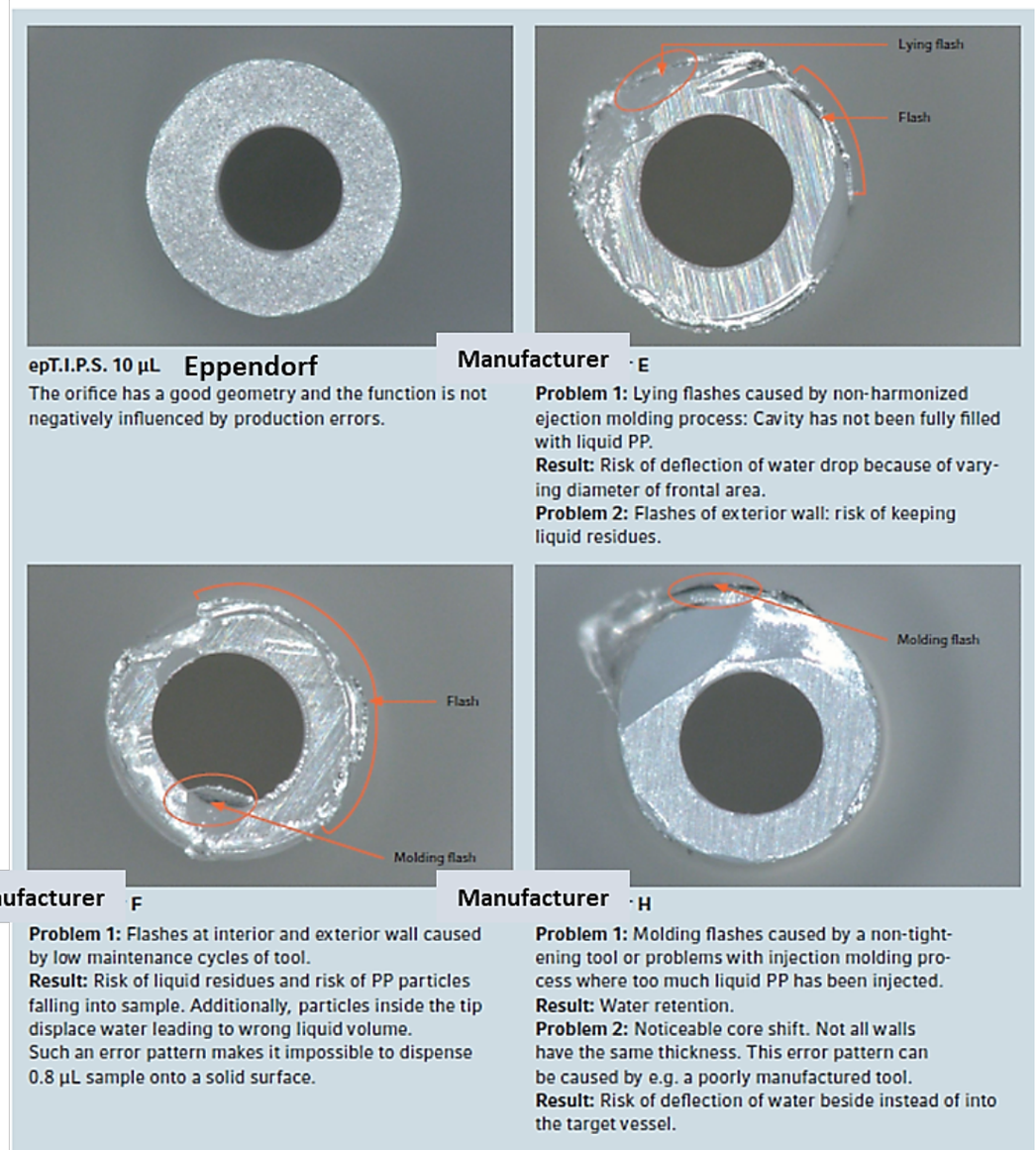

Figure 3. Microscopic images of 10 µL tip orifices by different manufacturers. Manufacturers E, F and H failed calibration at 1 µL by impaired systematic error. Source: Art *et al*., 2016

Sample retention

Sample retention in the tip, or “wetting”, is another factor that may affect pipetting precision and reproducibility. Tip wetting is influenced by the tip’s orifice, but mostly by its raw material and inner surface. This phenomenon is well known: when setting up a PCR or qPCR assay, a master mix is prepared with 10-20 % excess volume in order to compensate for pipetting losses due to retention of the liquid. Furthermore, since the effects of pipetting errors are cumulative, high sample retention can cause a pipetting system to be severely out of specification and thus diminish precision and reproducibility of any sensitive assay - in particular qPCR.

The predominant factors affecting sample retention in the tip are raw material composition and surface structure. These parameters vary considerably between different manufacturers and affect pipetting precision (Figure 4). The resulting qPCR assay variance may therefore strongly depend on which manufactures are used for pipetting the experiments. Sample retention is typically magnified when detergent (Figure 5) or glycerol-containing samples are pipetted: enzymes (polymerase) and various buffers or master mixes (Figure 6). This is due to the much lower surface tension and higher viscosity of these reagents as compared to water, which generates a stronger wetting effect on the inner surface of the tip. In addition to accuracy and precision being compromised (handling is often made even more difficult by the formation of foam), the loss of expensive reagents may be considerable, leading to increased assay costs – particularly in high throughput applications (qPCR, NGS).

One effective way to overcome this issue is to use low retention tips – for instance epT.I.P.S.® LoRetention (Eppendorf). These highly hydrophobic, plasma treated polypropylene tips retain considerably less residual liquid after use with various reagents, and they ensure improved reproducibility and sample recovery (Figures 5 and 6, Eppendorf LoRetention Video: https://www.youtube.com/watch?v=33BanWNFSnE). Compared to standard tips, this is a particularly valuable improvement when it comes to sensitive and cost-intensive applications like qPCR, PCR or NGS.

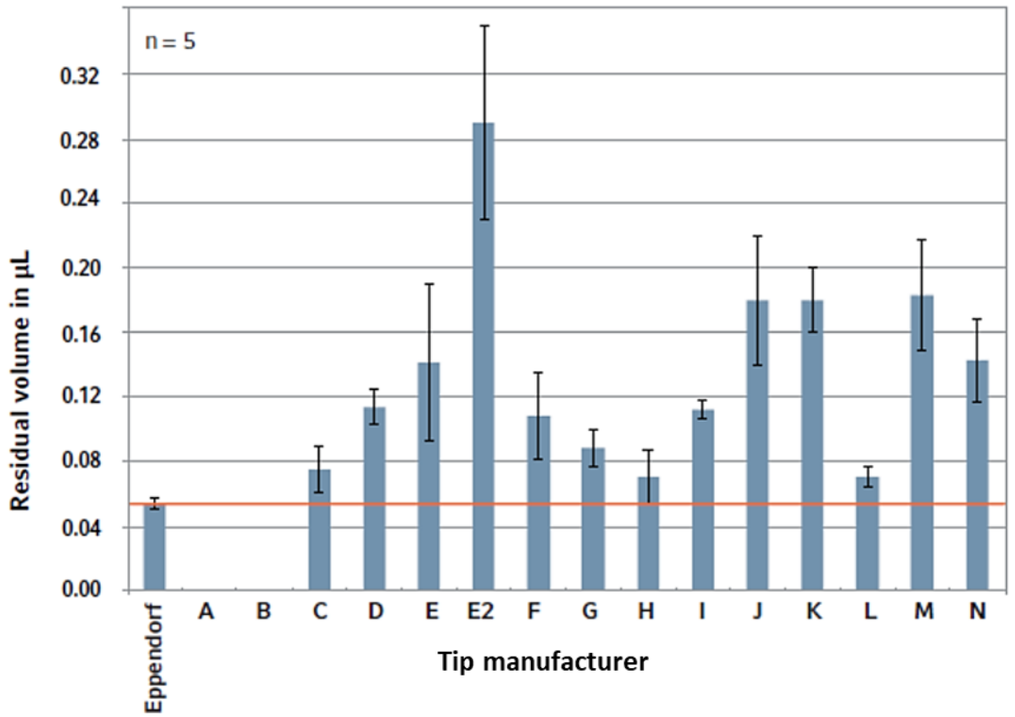

Figure 4. Sample volume retained in 1,000 µL tips by different manufacturers after pipetting 1,000 µL of water. Tips from manufacturers A and B did not fit tightly onto the pipette cone. Source: Art *et al.*, 2016

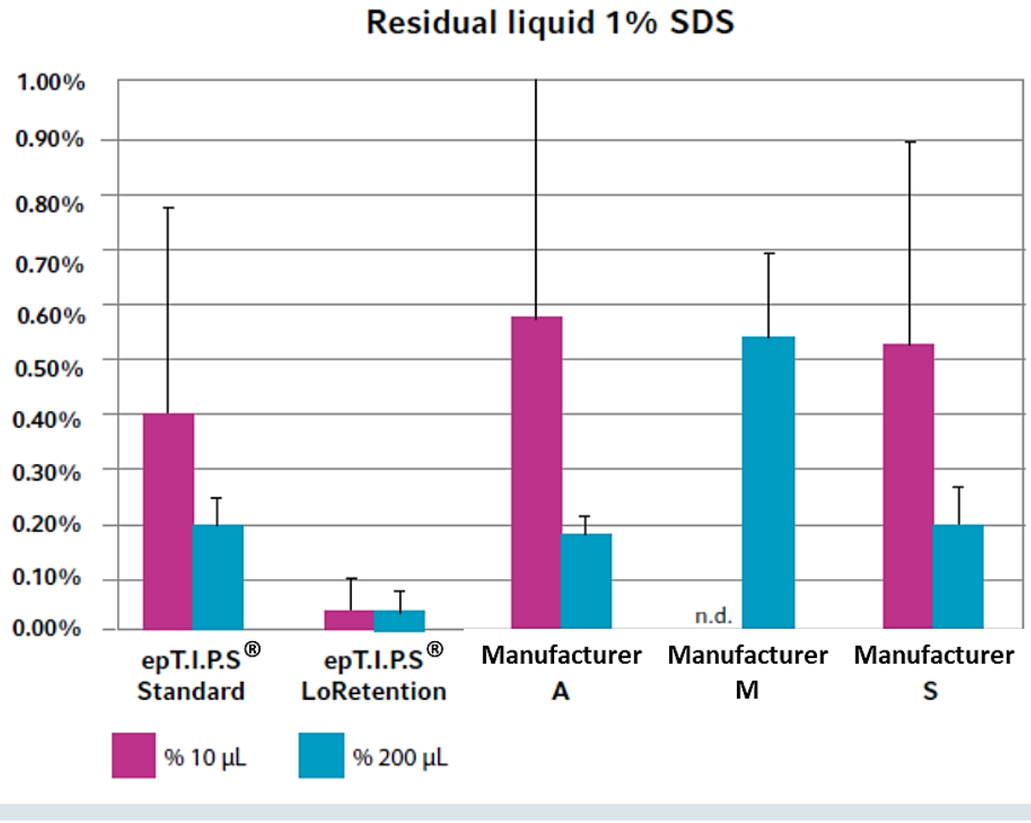

Figure 5. Determination of residual liquid using 10 µL and 200 µL pipette tips in combination with the electronic pipette Eppendorf Xplorer® for dispensing a 1% SDS solution (n.d.: not determined). Source: Weiss et al., 2018

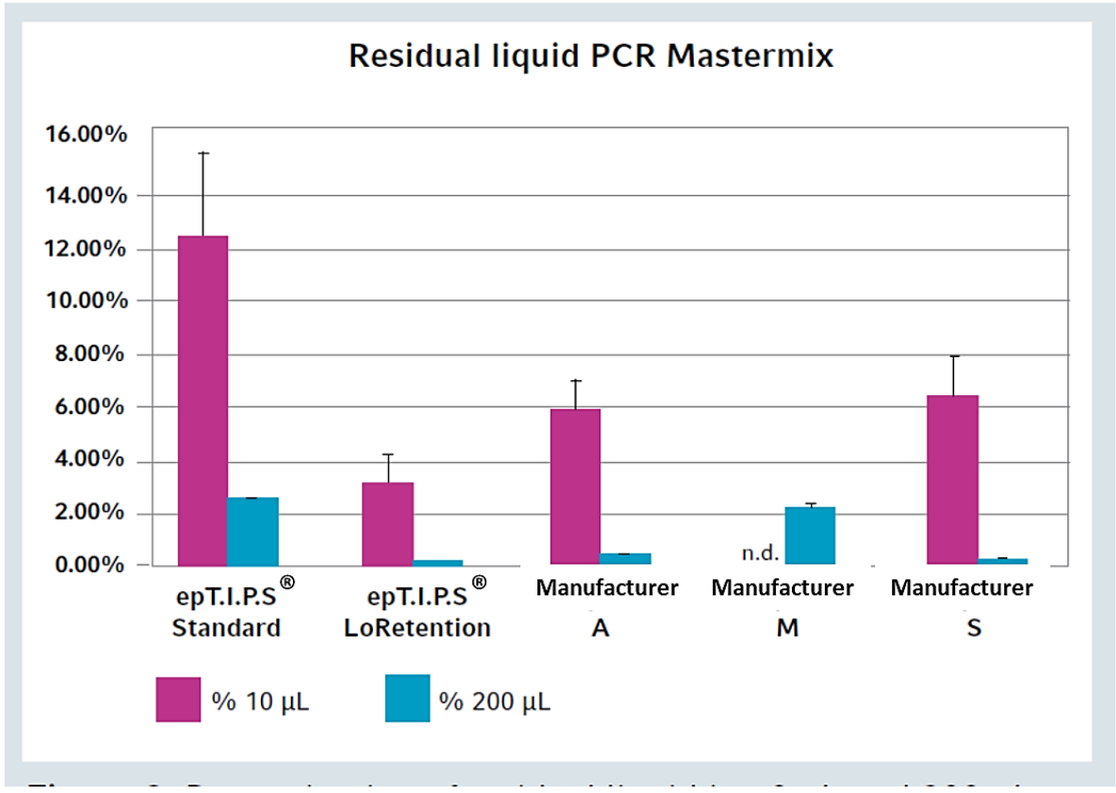

Figure 6. Determination of residual liquid in 10 µL and 200 µL pipette tips using the electronic pipette Eppendorf Xplorer® for the purpose of dispensing a PCR master mix (n.d.: not determined). Source: Weiss et al., 2018

The importance of a tight seal for pipetting precision and reproducibility

A basic requirement of a good quality tip is to fit tightly onto a pipette cone in order to generate an air-tight system. If this is not the case, precise aspiration and dispensing will be compromised, and leaking and partial sample loss may occur. This may introduce yet another variability factor to a sensitive assay such as qPCR.

It has been shown that pipetting errors may increase by up to 0.6 % (systematic error) and 0.8 % (random error), respectively, with the generic tips used (Carle et al., 2015). The ISO 8655-21:2002 indicates that the influence of ill-fitting pipette tips is as high as 0.5 - 50 % of nominal volume!

Manufacturers of pipettes and tips, also known as system providers, offer their customers a widely unnoticed additional service: they produce a system instead of individual parts. This means that the production tolerances of the pipette cone are aligned with the production tolerances of the tips and vice versa. Coordination of production tolerances is a feature which a non-system provider cannot achieve. Moreover, quality system providers (for instance, Eppendorf) typically comply with, ISO 8655-21:2002 and ensure (and certify) that the manufactured pipette/tip as a system fulfils the specified tolerances on the date of purchase. This is typically not the case with manufacturers which only produce tips – they do not have to comply with this requirement, and thus have the relative freedom of applying wider production tolerances. Tips from such providers may not fulfil required specifications and may therefore substantially weaken the performance of the pipetting systems with which they are used.

Autoclaving affects tips and pipetting performance

Users who need sterile tips have two options for achieving sterility: they either purchase sterile products or they decontaminate items by autoclaving. The first choice is safeguarded by professional quality assurance. High quality providers (*e.g.* Eppendorf) employ a specialized sterilization process which is validated, and sterility testing is performed by an external, accredited laboratory. The tests are performed in a batch-specific manner, and the resulting information is published in a lot-specific certificate available to the user online.

In the case of autoclaving, since conditions are not strictly controlled and may differ from the standard method (121 °C, 2 bar, 20 min), complete sterility is often questionable and, more importantly, is not validated afterwards. Furthermore, autoclaving may substantially impact tip geometry and the subsequent pipetting performance. Figure 7 shows that autoclaving influenced the dimensions of the pipette tips, with high inconsistency observed for various tip manufacturers and tip models. Compromised tip geometry impacts pipetting precision (Table 1), and therefore autoclaving should always be regarded as a factor that introduces additional variability to a given workflow. Laboratories using autoclaved tips should be aware of this influence, and it is highly recommended to check the performance of the pipetting system after autoclaving the tips. In case of high quality providers (Eppendorf), such a calibration is only needed if tips by other manufacturer are used. With Eppendorf tips, no negative influence on the calibration result was observed after the tips had been autoclaved (Figure 7).

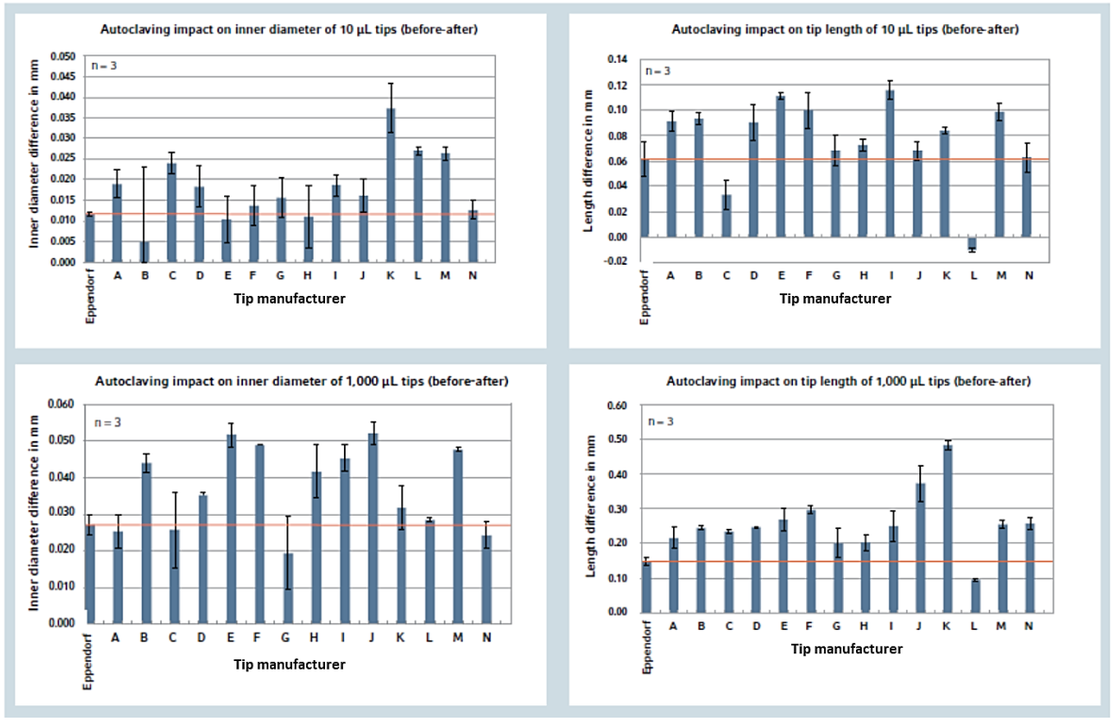

Figure 7. Dimensional changes of pipette tips from different manufacturers, induced by autoclaving.

	Before autoclaving							
	1,000 µL tip model				10 µL tip model			
	1,000 µL		100 µL		10 µL		1 µL	
Manufacturer	Systematic error %	Random error %	Systematic error %	Random error %	Systematic error %	Random error %	Systematic error %	Random error %
Eppendorf	V	V	V	V	V	V	V	V
A	/	/	/	/	V	V	X	V
B	/	/	/	/	V	V	V	V
C	X	V	V	V	V	V	V	V
D	V	V	V	V	V	V	V	V
E	X	X	X	X	V	V	X	V
F	V	V	V	V	V	V	X	V
G	V	V	V	V	V	V	V	V
H	V	V	V	V	V	V	X	V
I	V	V	V	V	V	V	V	V
J	V	V	V	V	V	V	V	V
K	X	V	V	V	V	V	V	V
L	V	V	V	V	V	V	V	V
M	V	V	V	V	V	V	X	V
N	X	V	V	V	V	V	V	V

	After autoclaving							
	1,000 µL tip model				10 µL tip model			
	1,000 µL		100 µL		10 µL		1 µL	
Manufacturer	Systematic error %	Random error %	Systematic error %	Random error %	Systematic error %	Random error %	Systematic error %	Random error %
Eppendorf	V	V	V	V	V	V	V	V
A	/	/	/	/	V	V	X	X
B	/	/	/	/	V	V	X	V
C	X	V	V	V	V	V	V	V
D	X no fit	X no fit	X no fit	X no fit	V	V	X	V
E	X	X	X	X	V	V	X	V
F	V	V	V	V	V	V	X	V
G	V	V	V	V	V	V	V	V
H	V	V	V	V	V	V	X	V
I	V	V	V	V	V	V	X	V
J	V	V	V	V	V	V	V	V
K	X	V	V	V	V	V	V	V
L	V	V	V	V	V	V	V	V
M	V	V	V	V	V	V	X	V
N	X	V	V	V	V	V	V	V

Table 1. Overview of changes in calibration results after autoclaving tips from different manufacturers. Grey marking: tips did not fit tightly onto pipette cone.

Leachables: the influence of plastic consumables on laboratory workflows

Leachables from lab consumables may affect various assay systems

qPCR and related workflows may be influenced by various factors. Typically, only the assay-specific factors are recognized as relevant and therefore controlled: sample material (quality, integrity, amount), reagent systems (quality, performance) and laboratory equipment (specifications, performance, quality).

Materials commonly used in medical and life-science lab consumables generally comprise a blend of base polymers, with a broad and heterogeneous range of chemicals added during the production process. These will facilitate or accelerate the processibility of the polymer, prolong its long-term stability or enhance its performance in the end-product in various ways. Increasing scientific evidence indicates, however, that a large part of those processing additives may be released (leach) from the consumable into the samples and pose a likely, and largely underestimated, source of error in various assay systems (Grzeskowiak, 2018; Grzeskowiak et al., 2015; McDonald et al., 2008) Leachables Webinar at LabRoots https://www.labroots.com/webinar/good-plastics-bad-plastics-how-tubes, Bioactive leachates from plastic consumables video: https://www.youtube.com/watch?v=LR1ljpp9TDE&list=PLCC61E0E91A68DDE3&index=18). These include enzymatic (McDonald et al., 2008; McDonald et al., 2009; Olivieri et al., 2012) and receptor binding (McDonald et al., 2008; Watson et al., 2009), as well as commonly used photometric assays. It was also reported that PCR assays may be affected

by leaching: several brands of PCR tubes were found to leach light-absorbing chemicals after exposure to a standard 30-cycle program on a PCR thermocycler (Figure 8) (Lewis et al., 2010).

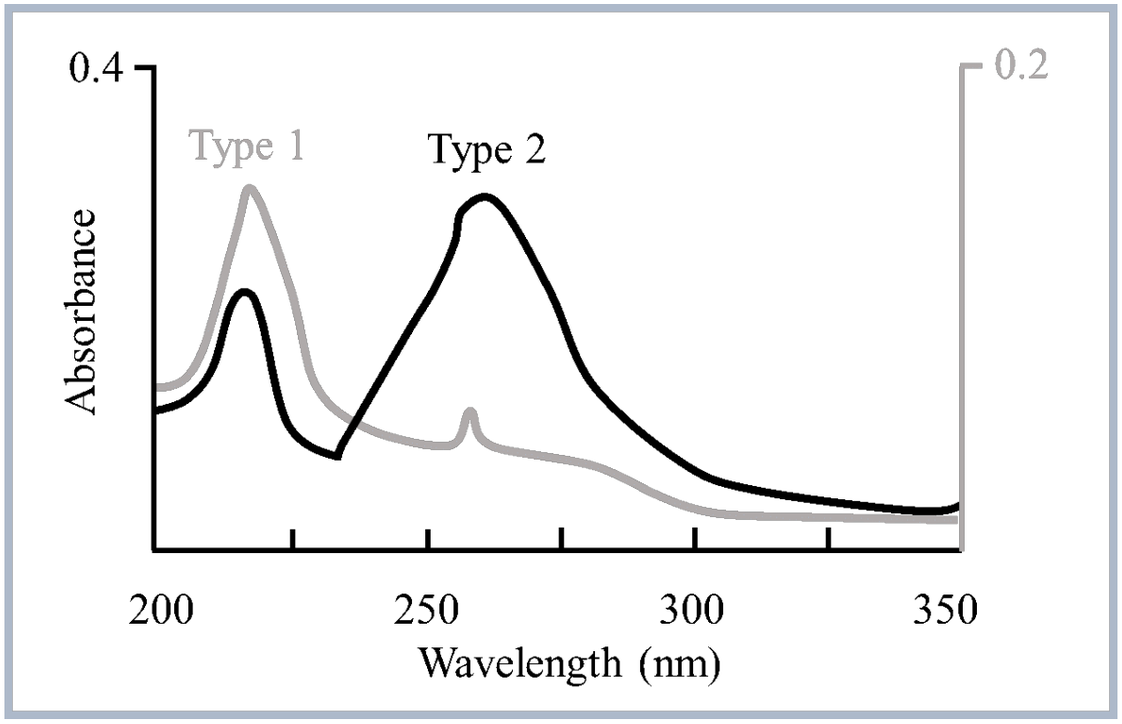

Figure 8. Substantial leaching of UV-absorbing chemicals from 0.2 mL PCR tubes into water after 30 cycles of PCR (30 s at 95 °C, 30 s at 60 °C, and 2 min at 72 °C). Two types of spectra generated by leachables, which strongly resemble nucleic acids, are shown. Source: adapted from Lewis et al., 2010.

The leaching process may vary greatly depending on experimental conditions, and different chemicals may be present in the sample in different amounts, thus exerting a wide range of effects on the assay (Olivieri et al., 2012). These effects are particularly relevant for plate-based assays, where a high variability in leachable levels has been observed in different wells, indicating position-dependent influences on the assay (Figure 9). In addition, cumulative effects have been observed with increasing numbers of pipetting or sample handling steps, demonstrating that even short contact times with the sample may lead to the release of relevant amounts of bioactive contaminants (Olivieri et al., 2012; Watson et al., 2009). Recent scientific publications on leachables and their bioactive effects are summarized in Table 2.

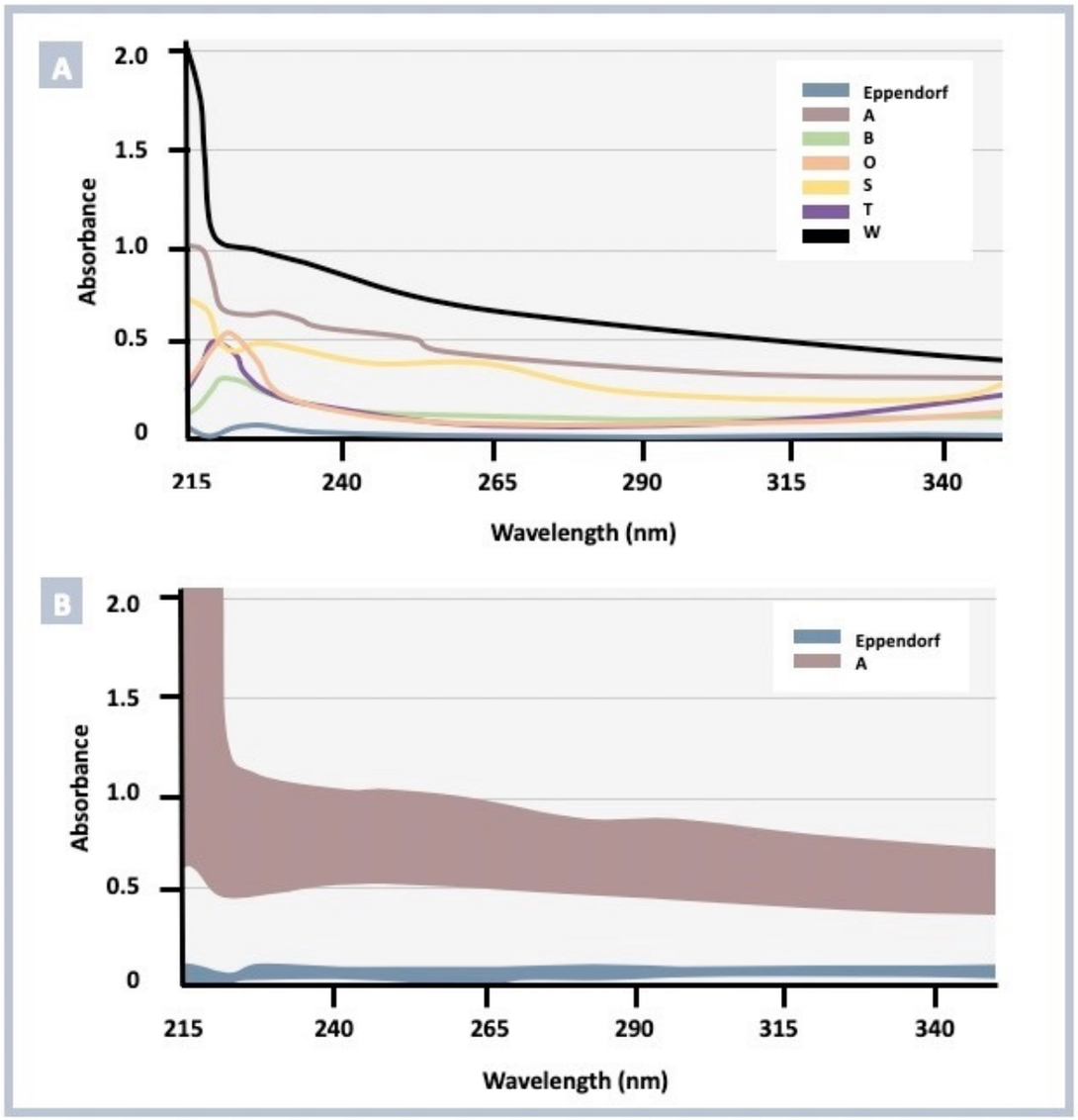

Figure 9. High levels of UV-absorbing leachables, measured in deep-well plates from various manufacturers. Deep-wells from different manufacturers were filled with 1 mL of analytical-grade water and incubated at 95 °C for 30 min. Samples from 96 wells were pooled (A) or collected from each individual well (B), and their absorbance spectra were assessed using a spectro-photometer. A) Average spectra obtained from 3 independent plates. High average leachable levels can be observed for most manufacturers. B) Variance of absorbance spectra of 96 individual wells in deep-well plates from two manufacturers. High variance of leachable levels between wells can be observed. Source: Eppendorf AG internal data

Publication	Consumables	Leachable	Type	Affected Assay
McDonald G.R. et al., Science 322, 917 (2008)	Tubes	DiHEMDA	Biocide	Altered hMAO-B (human monomine oxidase-B) activity
		Oleamide	Slip agent	
		unknown	Surfactant	Inhibition of $GABA_A$ (γ-aminobutyric acid type A) binding
Reid G. et al., GIT Laboratory Journal 9-10, 2-4 (2009)	Tubes	unknown	Surfactant	Inhibition of binding to GABA-A receptors
	Tips	unknown	Unknown	Inhibition of hMAO-B (human monoamine oxidase-B) activity
	Tubes	Oleamide	Slip agent	
Watson J. et al., J Biomol Screen 14(5), 566–572 (2009)	Tips	Erucamide	Slip agent	Alteration in G-protein-coupled receptor assay
Lewis, L. K. et al., BioTechniques 48, 297-302 (2010)	Tubes	Mixture of small molecules (200 - 1400Da)	Unknown	Increased photometric readings at 220 nm and 260 nm affecting DNA/RNA and protein quantification
	PCR tubes			
Hammond M. et al. Biotechnol Prog. Apr: 332-7 (2014)	Single-use bioprocess equipment	bDtBPP Butylphenyl-phosphate	Antistatic stabilizer	Reduced growth of cell cultures
Birch NP. et al. J Neurochem. Apr; 133(1) (2015)	Syringes, tubes	stearic acid, palmitic acid, 1,2-ethanediol, unknown	Plasticizers	Alterations and growth reduction of cultured neurons

Table 2. Bioactive effects of leachables - literature review

Minimizing the effects of leachables

In recent years, increasing evidence of consumables distorting experimental results has changed the view of the scientific community; high quality consumables and comprehensive information on critical additives provided by the manufacturer are becoming increasingly important (McDonald et al., 2009; Lee et al., 2015; Schiffer et al., 2014; Cressey, 2008).

There are several steps researchers can take to minimize the risk of leachables affecting their experiments (McDonald et al., 2009; Olivieri et al., 2012; Grzeskowiak et al., 2015). Some preliminary basic steps may involve simply taking inventory of consumables used in the laboratory to gain an overview of the situation and thus make it easier to react when problems occur. Standardization may also prove a good way to minimize the effects of leachables: consistently using consumables from one manufacturer and avoiding changing or mixing them between series of experiments. Furthermore, using consumables from a manufacturer that provides/guarantees high consistency across consecutive production lots is essential when attempting to minimize technical variances. This is especially important for assays that require the use of consumables from different lots, e.g. long-term routine experiments or high throughput assays. For assays known to be sensitive to leachables, each new production lot purchased should be ordered early enough and be tested for any differences against the current batch within the same

experimental setup. Finally, it is important to choose consumables from manufacturers who comply with high quality production criteria (clean room conditions, high degree of automation) and provide consumables made of virgin polymers and strictly free of the additives described in the literature to be critical: slip agents, biocides and plasticizers (Table 2, Olivieri et al., 2012), Leachables Webinar at LabRoots https://www.labroots.com/webinar/good-plastics-bad-plastics-how-tubes). Furthermore, strict testing of each individual production lot, as well as external examination and certification of every lot comprising an advanced purity grade, are essential steps in the process of monitoring consistently high product quality. Eppendorf, as a premium consumables manufacturer, is in compliance with all these standards which results in conscious high production and final product quality maintenance (http://consumables.eppendorf.com/). A comprehensive certification of quality standards, which includes the absence of avoidable additives, is provided for all consumables as well as lot-specific certification for advanced purity grades (www.eppendorf.com/certificates).

In summary, an awareness of consumables and leachables as potential sources of interference with the laboratory workflow, coupled with appropriate laboratory routines and strict avoidance of consumables not certified to be free of the critical additives, provides a best practice for minimizing the risk of leachables affecting the experiments.

Low binding consumables: maximizing sample recovery and assay performance

Preparation and storage of small amounts or highly diluted samples of nucleic acids and proteins is a critical step and may significantly influence the results of qPCR (and other sensitive applications) in various fields: molecular biology, forensics, analytics and bio-pharma. Sample quantity and purity are typically functions of biological material quality and availability, preparation and handling methods, but also of the consumables used (Goebel-Stengel et al., 2011). Non-specific adsorption of bio-molecules (nucleic acids and proteins) to the polymer surface of consumables has been shown to be a substantial factor contributing to sample loss during storage and handling in various lab applications (Kristensen et al., 2015; Hoofnagle et al., 2016; Kraut et al., 2009). This process is largely conveyed by non-specific binding of hydrophobic domains in nucleic acids and proteins to the hydrophobic polymer surface, leading to both structural denaturation and activity loss as well as to an abrupt decrease in sample concentration: up to 90 % of sample may be adsorbed within 6 hours of storage (Figure 10) (Weiß et al., 2016).

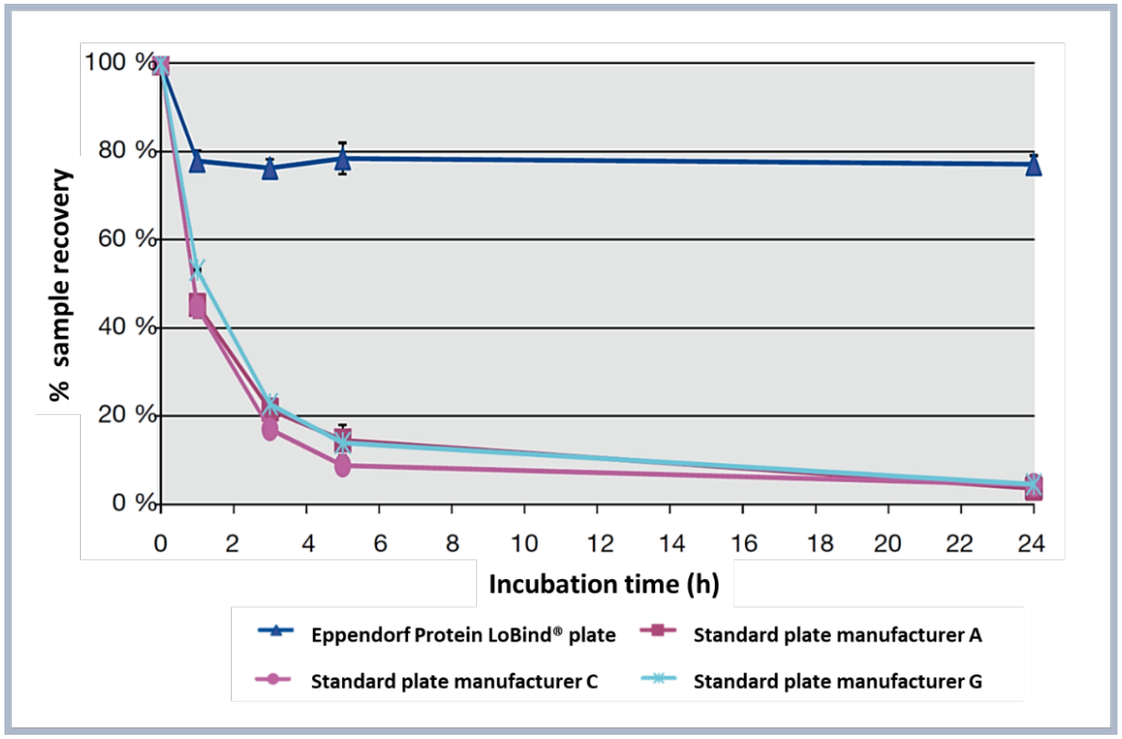

Figure 10. Recovery rates of protein samples (fluorescein-labeled BSA) after incubation in 384-well deep-well plates with a total volume of 200 µL. Source: Weiß et al., 2016.

Several manufacturers of lab consumables approached this problem through various material modifications. The most common method is coating (siliconization), which is designed to create a barrier between biomolecules and the polymer surface and thus diminish binding. Depending on experimental conditions, however, the coating may be not sufficiently stable and migrate into the sample (leaching), which can have adverse effects on sample purity and experimental results (Smith et al., 2011; Bowen et al., 2014). Another approach uses a low retention modification of the surface, which decreases sample retention during liquid handling steps but per se does not lead to decreased adsorption at the molecular level. A third approach comprises direct material optimization, where the hydrophobicity of the material is reduced (Eppendorf LoBind®), leading to reduced adsorption of bio-molecules to the surfaces of consumables (Figure 11, Eppendorf LoBind Video: https://www.youtube.com/watch?v=zcRu1XEfoDU&list=PLCC61E0E91A68DDE3&index=5). Notably, these three approaches to low binding materials rely on very different molecular mechanisms, and respective sample recovery rates may differ greatly between various low-binding consumables (Figure 12).

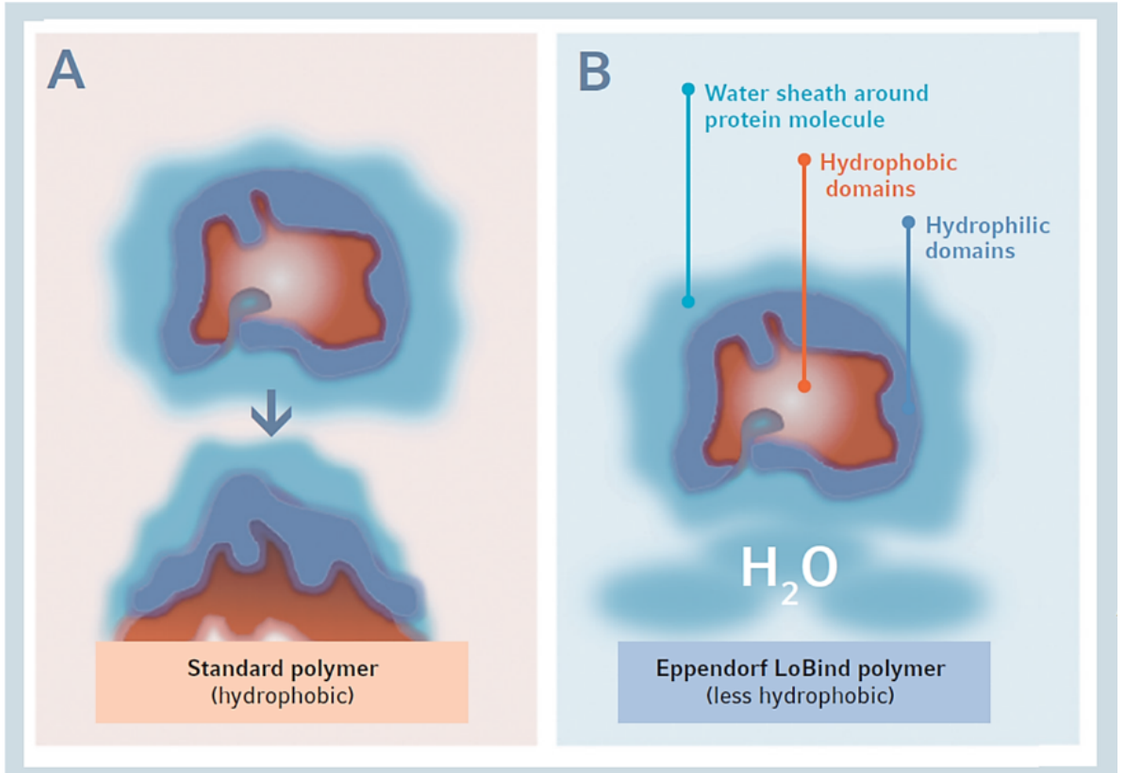

Figure 11. A) Standard polypropylene: bio-molecules (proteins or nucleic acids) bind to a standard polymer surface via hydrophobic domains. This results in structural denaturation and loss of activity/concentration. B) Eppendorf LoBind® Polypropylene: water molecules migrate between polymer and protein molecules and prevent binding to the surface and denaturation.

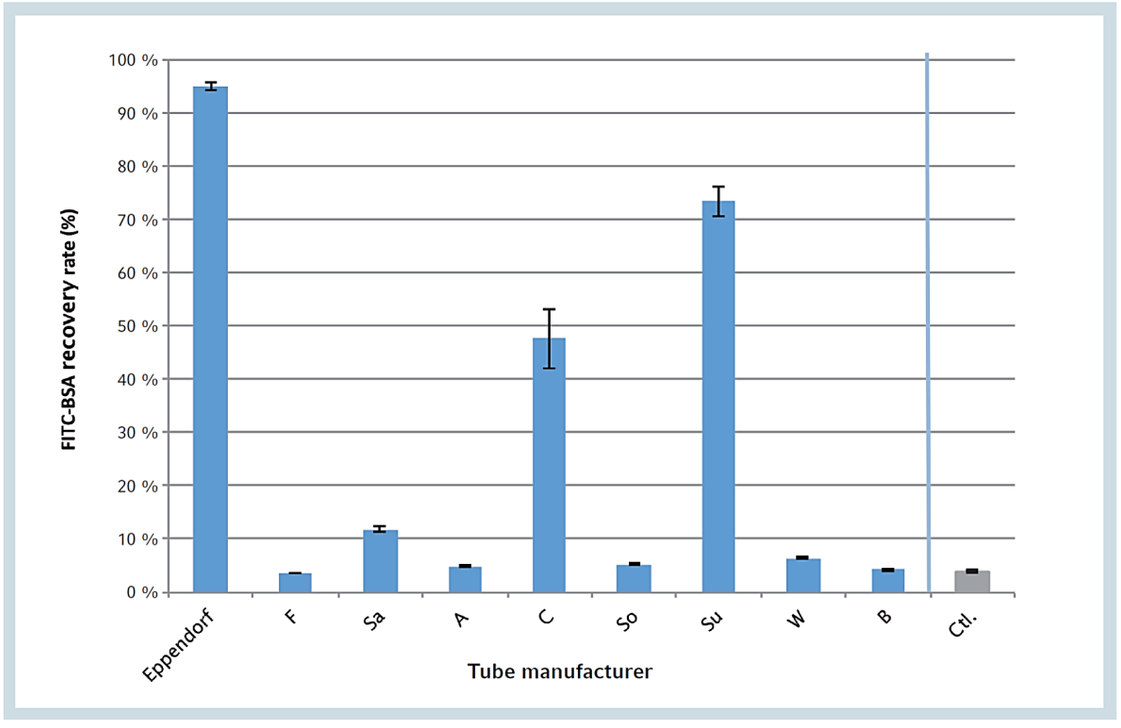

Figure 12. FITC-labeled BSA recovery rates after 24 hours of incubation in 1.5 mL low binding tubes from different manufacturers. Negative control (Ctl.): standard polypropylene tubes. Two independent experiments were performed in triplicate (n=6). Source: Grzeskowiak et al., 2016.

Non-specific sample binding and subsequent loss of activity may be critical factors influencing experimental results, particularly in the case of sensitive methods/assays like qPCR, or when limited sample amounts are used in diagnostics, forensics or analytics. By employing high-end low binding consumables during sample preparation and assay setup, the resulting maximization of sample recovery may improve the efficiency, sensitivity and overall performance of PCR and qPCR assays. One of the

examples includes augmentation of real-time PCR efficiency by preparing serial dilutions of standard panels and storing them in Eppendorf DNA LoBind PCR Tubes (Lecerf et al., 2010). Also, complex protocols involving multiple PCR and purification steps, such as Next Generation Sequencing (NGS), may benefit significantly from low binding consumables: a higher average number of reads, as well as a broader variation of transcript species can be achieved, even when working with libraries of low quality or low amounts of starting material (Henke et al., 2016; 2).

Another critical step during which sample loss may occur is mid- to long-term storage. Here, low binding consumables may maintain stable recovery rates of DNA samples after weeks or even months of storage and thus assure stable performance of the qPCR assay (Figure 13) (Henke et al., 2016; 1).

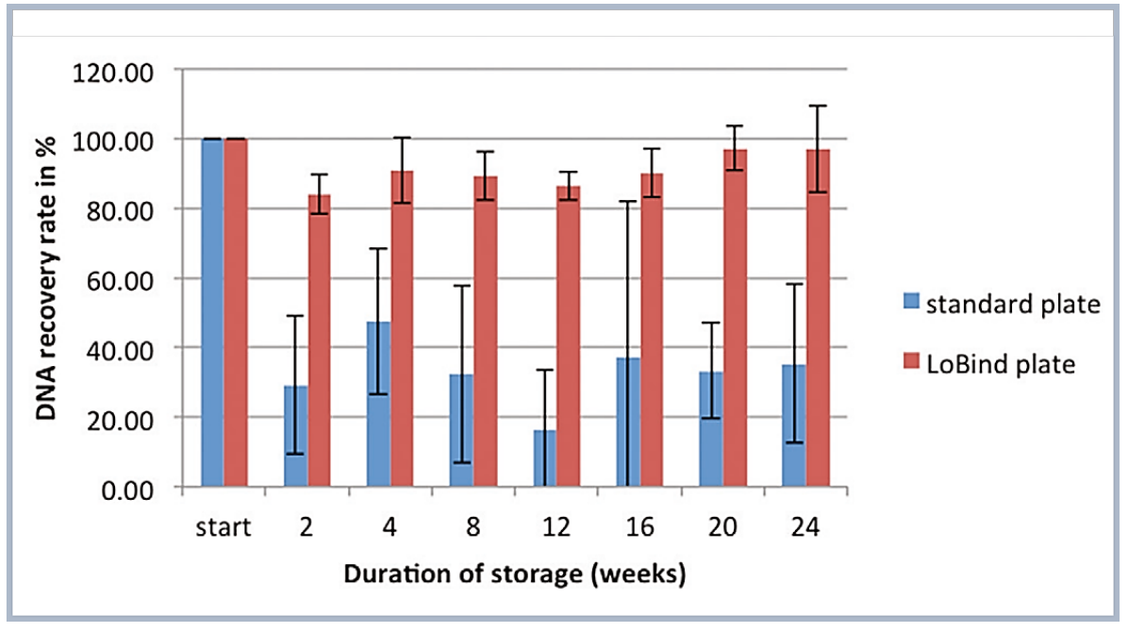

Figure 13. qPCR experiments with libraries of low starting amounts of human genomic DNA: percentage of DNA recovered in standard and LoBind PCR plates after storage at -20 °C. Five replicates of each library were analyzed. The diagram shows the average DNA recovery in % of three different libraries. Source: Henke et al., 2016; 1.

In summary, the use of high quality low binding consumables during sample preparation and storage, as well as assay setup, allows substantial improvement of experimental performance in the qPCR workflow and should always be considered when working with limited amounts of sample material.

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Chapter 8

qPCR Assay Validation

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Section 1

Introduction

Quantitative PCR (qPCR) is the technique of choice for many DNA quantitation studies that provides affordability, precision, and necessary accuracy across a wide dynamic range. This is specifically true for applications such as gene expression, bacterial/viral load measurements, allelic discrimination, and species identification. It is a technique that is easy to execute and can provide rapid quantification of nucleic acids. Moreover, qPCR remains the gold standard for many applications, such as detection of mRNA (Bustin, 2000, Bustin, 2002). Because of its broader linear dynamic range and greater sensitivity, qPCR is used to reanalyze changes in gene expression suggested by exploratory technologies such as microarrays and RNA-Seq. However, imperfections such as variable pre-assay conditions, poor assay design, and improper data analysis have led to inaccurate results and erroneous conclusions from qPCR experiments (Johnson, et al, 2014).

The Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines were published with the aim to provide a framework that researchers could follow to prevent publication of inconsistent, inaccurate, and misleading data that is populating and corrupting the peer-reviewed literature (Johnson, et al, 2014, Bustin et al, 2009). Although the rationale for following the MIQE guidelines has been widely accepted, the numbers of publications not following the guidelines still vastly outnumbers publications

following them (Bustin et al, 2013). A properly optimized and validated assay is one of many prerequisites of the MIQE guidelines for obtaining accurate, precise, and reproducible data in qPCR. Frequently, researchers do not validate primers they acquire from peer-reviewed publications, other lab members, or from vendors. They make the assumption that researchers who published qPCR data using these primers validated them from the same type of material (the specific cells or tissues) of their current study, but this is often an incorrect assumption (Wang et al, 2012). Frequently, the primers procured from vendors have been designed in-silico with validation data not provided which does not conform to the MIQE guidelines (Bustin et al, 2011). Given that data quality and accuracy is entirely dependent on the predictable behavior of primers in each reaction, newly acquired assays should be fully validated for annealing temperature, specificity, reproducibility, efficiency and linear dynamic range using a representative DNA sample (Taylor et al, 2010, Mikeska et al, 2009). A qPCR instrument with thermal gradient capability will greatly facilitate these validation steps. Moreover, validation of primers is specific to sample type (cell lines or tissue types for example), RNA extraction method (column-based extraction versus TRIzol for example), type of supermix, and reverse transcriptase (RT) kit used. Independent primer validation should always be performed when any of these parameters are changed to avoid risking inaccurate gene expression results and eventually incorrect experimental conclusions (Opel et al, 2010).

The choice of using primer or probe based assays depends on multiple factors including nature of application, sequence and region of the gene of interest in the genome, number of targets to be detected, and the abundance of the gene of interest. If probe based chemistry is used in a qPCR experiment, validation of the primers should be done first to ensure the absence of amplification artifacts affecting the kinetics of the assay (Bustin et al, 2004).

Thermal gradients for determination of annealing temperature

Annealing temperature is an important step in a PCR reaction and is one of the first validation steps that should be performed when new primers are evaluated. Optimal annealing of the primers to their target is critical determinant of amplification efficiency. To determine the proper annealing temperature, a 10°C to 15°C temperature gradient using a gradient enabled thermal cycler should be run around the theoretical Tm of the primers (approximately ±6°C around the theoretical Tm). DNA template used for this first validation step could be originating from plasmid, gel isolated PCR product, genomic DNA (gDNA) or complementary DNA (cDNA). However, it is recommended to use a pooled DNA template originating from the samples derived from different treatments or conditions of a project (Taylor et al, 2010). The objective of a thermal gradient is to obtain a temperature range that will generate amplification curve resulting in the lowest quantitative cycle (Cq) value without generating secondary products or primer-dimers. As shown in Fig 1, the optimal annealing temperature range of the primers in this example is between 55-58°C. When possible, using identical annealing temperature for the different primers in a typical qPCR project is highly recommended and permits running multiple primer pairs on the same plate during a qPCR experiment. Using consistent annealing temperature can also permit multiplex qPCR assays using probes with different fluorochromes.

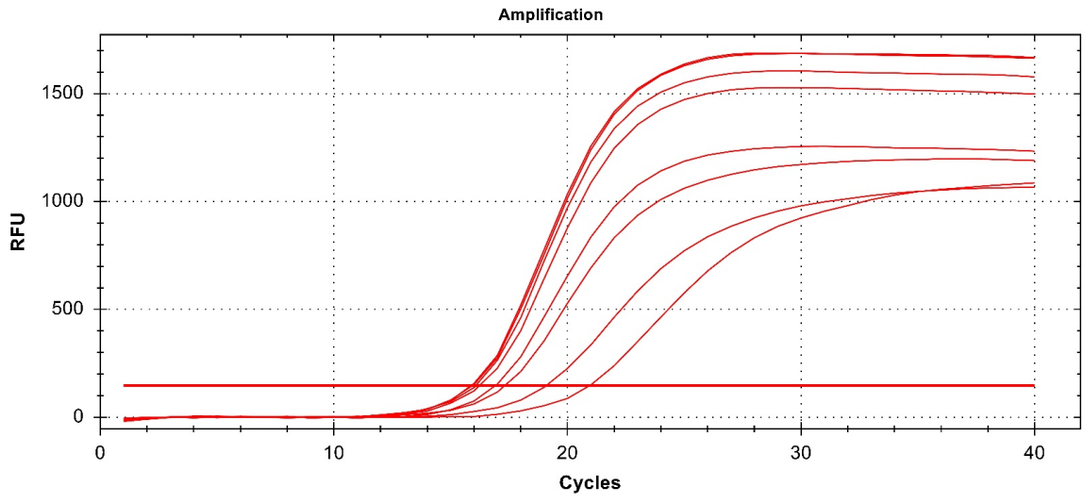

Figure 1. Validation of primers annealing temperature using a thermal gradient. In order to find the best annealing temperature range for a primer pair, a thermal gradient from 55°C to 70°C was run. Temperatures in the range of 55-58°C gave the lowest Cq value compared to temperatures in the range of 61-70°C.

1.1 Melt Curve

Melt curves determine assay specificity by assessing the temperature dependent dissociation of double-stranded DNA. Running a melt curve ensures single products have been amplified in a qPCR reaction when using an intercalating fluorescent dye such as SYBR Green or EVA Green. To create a melt curve, the change in fluorescence intensity is plotted against gradual increase in temperature. Typical melt curves run approximately between 65-95°C with 0.5°C increments, and a 5 second hold at each temperature to read fluorescence intensity. A sudden drop of fluorescence intensity will be observed when the amplicon Tm is reached which is specific to the amplicon length and GC content. The first order derivative of a melt curve plot is typically represented in most qPCR software such that the melt profile gives a peak at the specific melting temperature of a given amplicon. Multiple or shifted peaks in a melt curve can indicate problems associated with primer specificity, cross DNA contamination, primer-dimers, or genomic DNA contamination. When these double stranded non-specific products are amplified in a reaction, the intercalating dye will bind to them and this leads to increased fluorescence intensity from the qPCR reaction, resulting in increased reaction efficiency over 100%. In turn, this leads to lower than expected Cq values and an over estimate of target DNA, which will affect accuracy of results and conclusions. Therefore it is critical to run melt curves on unknown samples, no template control (NTC) controls, no reverse-transcriptase (NRT)

controls, as well as positive and negative control samples to ensure single peaks are detected.

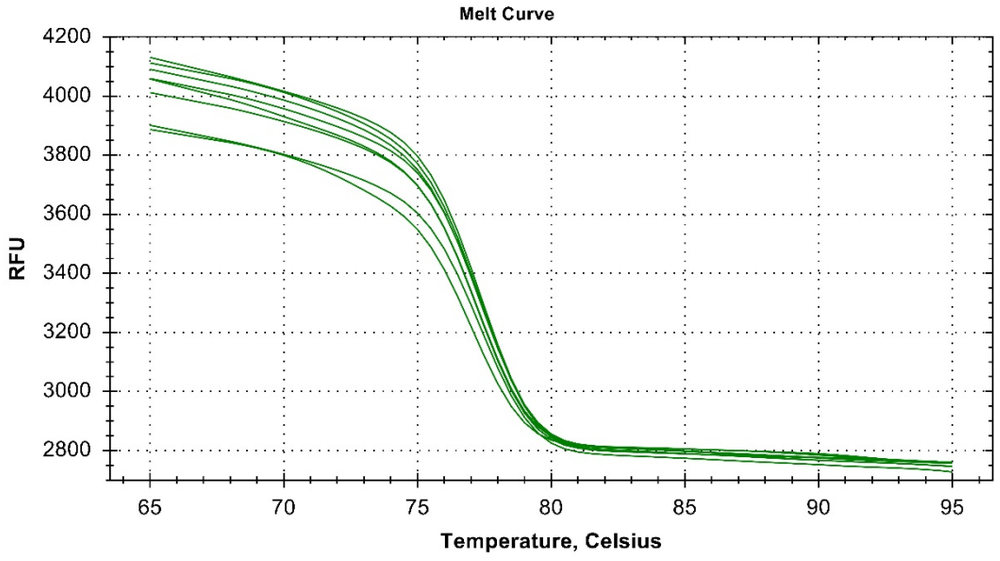

Figure 2. Melt curve analysis. A) A melt curve analysis is run to determine the melting temperature (Tm) of PCR products. B) A single melt peak is necessary to ensure that only one product is amplified by the primer pair and that no primer-dimer is produced. Creation of secondary products or primer-dimer can alter qPCR efficiency and will affect accuracy, precision, and reproducibility of results.

1.2 Gel Analysis and Amplicon Sequence

It is highly recommended to run a sample of a qPCR reaction on an agarose or polyacrylamide gel when validating a primer pair – A polyacrylamide gel will generally resolve smaller fragments than an agarose gel (Fig 3). This will confirm that only one product is amplified per primer pair and that the size of the amplified amplicon is in concordance with the theoretical amplicon size. Finally, the PCR product should be sequenced in order to confirm that the primers definitely amplify the amplicon of interest. This will avoid the situation where primers might amplify a product unrelated to the original design, especially if the primers were not blasted to confirm that they only anneal to the gene of interest or when working in a partially or unsequenced genome.

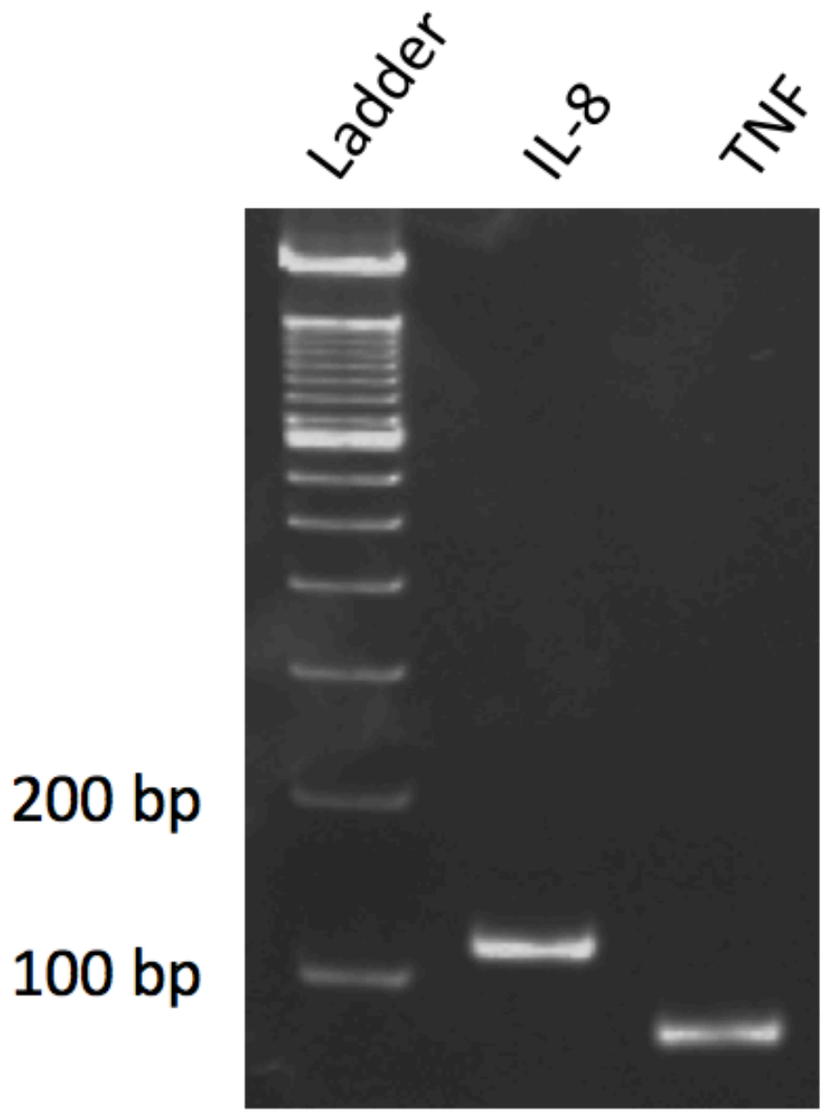

Figure 3. Image of amplicons amplified from qPCR assays. Amplified products of the PCR reactions were loaded on a 10% acrylamide gel to verify if the amplicon size corresponds to the theoretical amplicon length. The lack of secondary products confirms that a primer pair is only amplifying one amplicon.

Section 3

Standard curves

The standard curve is created by plotting the logarithm of the DNA initial starting quantity on the x axis and the Cq values obtained on the y axis. The purpose of a standard curve will depend on the quantification method desired. Standard curves for absolute quantification are run primarily to assess the absolute concentration of a gene of interest in a sample. For this purpose, purified target DNA samples (such as plasmid DNA, extracted PCR product, or DNA spiked in a sample) should be used to generate a standard curve. A serial dilution of the standard is then run on a plate alongside unknown samples. The resulting Cq of the unknown samples are then fitted to the standard curve to measure the absolute concentration of desired sample (copies per µl for example). A standard curve should be run on every plate for absolute quantification experiments.

Standard curves are also used to determine amplification efficiency, reproducibility, and dynamic range of each qPCR assay. This primer validation process is necessary prior to using each new primer set in a qPCR experiment. Primer validation is performed regardless of absolute or relative quantification in qPCR. An 8-point serial dilution done in technical triplicates (excluding the NTC) is usually recommended for validating primers when doing a standard curve (Fig 4). A precise standard curve with tight technical replicates (Cq standard deviation of the technical replicate < 0.25) is highly desirable (Fig 5). Ideally, the template used for assessing the standard curves would be a pooled DNA template coming from

different samples/conditions of a project. The dilution used for generating the standard curve will be such that the last point of the standard curve should have no amplification or almost no amplification of the target (Cq > 35). This is done to ensure that the curve covers all the possible template concentrations that could be encountered during the qPCR experiment to produce the largest dynamic range for the assay.

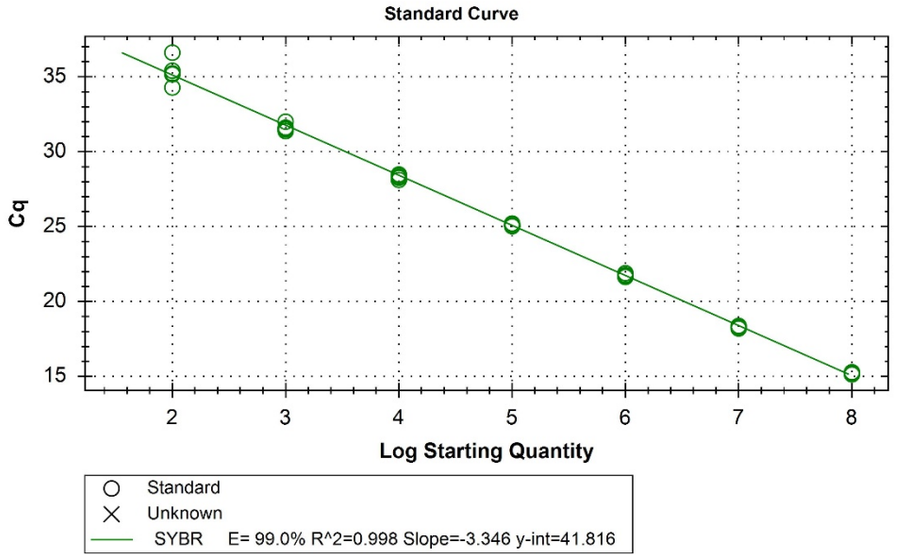

Figure 4. Standard curve. A 7-point qPCR standard curve was generated from a 1 in 10 dilution of a DNA sample using a primer pair . From the measured slope (m = -3.346), the qPCR efficiency was calculated (E = 99.0%). The fitness of the data to the standard curve was also assessed by evaluating the R2 value (0.998). The primer pair dynamic range for the PCR amplification was also assessed with a Cq range of 15 to 35.

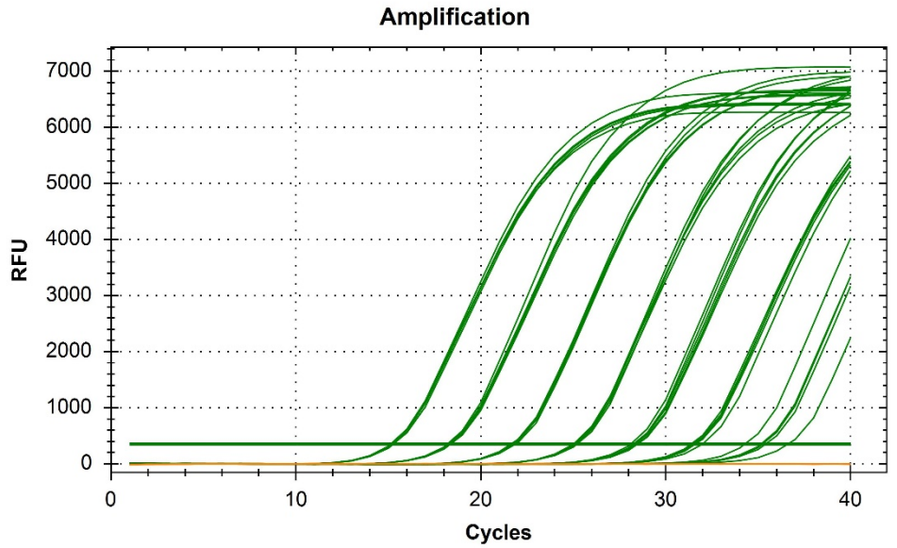

Figure 5. Reproducibility of the standard curve. Tight technical replicates with a standard deviation < 0.25 is highly desirable when generating a standard curve. Stochastic effect of qPCR amplification for rare targets can be seen when Cq > 30. This results in poor reproducibility of technical replicates with associated standard deviation > 0.25. The no template control (NTC; orange line) shows no amplification, indicating the absence of primer-dimer formation.

2.1 PCR amplification efficiency

The efficiency of a qPCR reaction is the degree at which the polymerase will convert reagents to produce an amplicon of interest. Many factors can affect the efficiency of a qPCR reaction such as amplicon length and sequence, primer sequence, impurities and inhibitors in the sample, and cycling conditions. The determination of the efficiency of a qPCR reaction is especially important if one is using the ΔΔ Cq calculation method (Pfaffl, 2001). Once the standard curve is generated, the efficiency of a qPCR reaction is then calculated from the slope of the log-linear portion of the standard curve (10-1/slope-1) (Bustin et al, 2009). The maximum rate of increase of an amplicon at every cycle is two-fold, which corresponds to an amplification efficiency of 100%. An optimized qPCR assay should have efficiency between 90-110% (Taylor et al, 2010). Both the target of interest and reference gene(s) used in a qPCR project should have optimal amplification efficiency. Moreover, the fitness of the data to the standard curve (how linear the data are) should also be assessed by evaluating the r or R2 value. Linearity of the data will indicate variability of Cq values across technical replicates of the standard curve. Ideally, an r value > 0.99 or an R2 value >0.98 is deemed desirable for a qPCR assay (Taylor et al, 2010). Noteworthy differences between the Cq values of the standard curve technical replicates will lower the r and R2.

A poorly designed primer that shows non-specific binding and off target amplification will increase the amplification efficiency since additional fluorescence will be generated when using a non-specific fluorescent dye such as SYBR Green. Furthermore, formation of primer dimers and presence of contaminants might lead to increased amplification efficiency as previously discussed. Conversely, a primer that does not display 100% complementarity to the target mRNA will reduce amplification efficiency below accepted levels. Presence of secondary structures such as poly A tails in the amplicon can also cause the amplification efficiency to drop (Johnson et al, 2014).

2.2 Linear dynamic range of the standard curve and diluting samples per primer pair

The linear dynamic range of a standard curve is the range where the qPCR assay is deemed linear. This dynamic range should be assessed and would minimally cover 3 log10 of magnitude and preferably would broaden to 5 or 6 log10 concentrations. It might be necessary to delete points at one or both end of the standard curve to obtain an acceptable amplification efficiency and R2 value. This will then define the dynamic range of the qPCR reaction for a pair of primers across a range of DNA concentrations. It is crucial for any Cq value obtained from the qPCR experiment to fall within this linear dynamic range. If the Cq value for a particular sample using a pair of primer is lower than the lowest Cq value of the standard curve dynamic range, then

that sample should be diluted so that the Cq value falls within the dynamic range of the established standard curve. The linear dynamic range of a primer pair will also define the dilution factor that should be used for the DNA samples.

Given that a pooled DNA sample should be used as the starting point to define the standard curve, the middle point of the dynamic range of the standard curve should be targeted as the ideal dilution factor for every samples. For example, if the dynamic range for a primer pair is found to fall between Cq values of 20 to 30, a Cq value of 25 would be the target for the ideal dilution of the DNA samples. This would correspond to a delta Cq of 5 (25-20) and a dilution factor of 32 (25) would be recommended for that particular primer pair. It is thus possible that different dilutions of the same DNA sample will be used during a qPCR project for gene expression evaluation depending on the dynamic range of the different primer pairs that are part of a particular project.

Conclusions

RT-qPCR is a technique commonly used in many laboratories for gene expression analysis. However, strict procedures must be taken in order to ensure accurate and reproducible qPCR results. Primers that are custom designed should be validated according to MIQE guidelines. The same validation step should be performed on primer sequences taken from the literature as many published primer sequences have not been validated to MIQE standards. Validation of the primers is one of many important aspects of the MIQE guidelines that target the reliability of qPCR results in order to improve the integrity of the scientific literature, reproducibility of results between laboratories, and ultimately increase experimental transparency.

Section 5

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Chapter 9

Quality Control in qPCR

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Section 1

Introduction

Quantitative real-time PCR (qPCR) has developed into the central platform and gold standard for much of molecular research and molecular diagnostics. Behind this success is the development of routines for quality control, validation, and performance assessment. Recently a group of opinion leaders, coordinated by Professor Stephen Bustin, drafted the minimum information for publication of quantitative real-time PCR experiments "MIQE" guidelines that teach what information about assays and test performance shall be reported when submitting a scientific report for publication (Bustin et al, 2009), and the European Commission funded SPIDIA to generate routines and tools for quality control of the pre-analytical processes in molecular diagnostics, based on which the European Commission for Standardization (CEN) drafted nine new technical specifications that were launched during 2015, and the International Organization for Standardization (ISO) launched eight new projects within "Clinical laboratory testing and in vitro diagnostic test systems". The National Institute of Standards and Technology (NIST), have made standard reference materials (SRMs) available for genetic analyses and the Clinical Laboratory and Standards Institute (CLSI) has developed guidelines and protocols to validate tests' performance. These tools and methods are now available to the researchers through providers such as TATAA Biocenter and MultiD Analyses, whose GenEx software is today compatible will qPCR instruments from all the leading manufacturers. Forums dedicated to qPCR data analysis and quality control has also appeared. In this chapter we shall

discuss the most confounding aspects of molecular diagnostics and present the tools available to deal with them.

Section 2

Testing the RNA quality and post sampling response

Sampling is arguably most critical for accurate molecular analysis and there are several challenges. One common complication is sample heterogeneity, with many different cell types being present is a sample and many of the cell types are themselves heterogeneous in terms of expression profiles (Kubista, 2015). This can be addressed by single cell profiling (Bengtsson, 2005), and detailed step-by-step protocols are now available (Stahlberg, 2014). Another complication is that upon sampling the cells' environment changes dramatically, inducing a molecular response with many genes being activated, while others are repressed (Malentacchi et al, 2014).

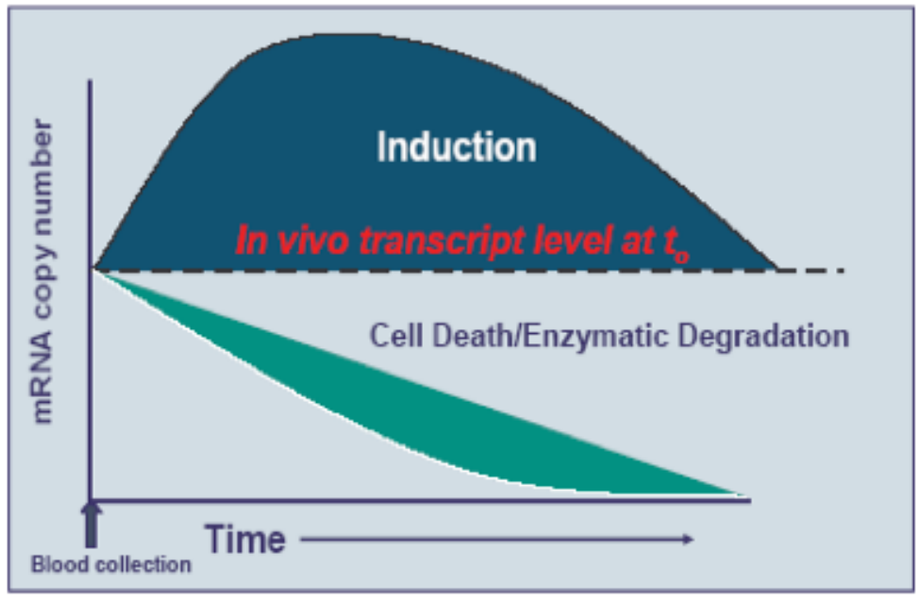

Figure 1. Schematic showing post sampling effects on mRNA levels.

For very small samples, from a single to a few thousand cells, direct lysis reagents have been developed, such as the Cellulyser (Tataa), that immediately lyse the cells, hence inactivating RNA metabolism, and stabilize the RNA. The direct lysis reagents are compatible with downstream molecular analyses such as RT-qPCR, allowing quantification of the transcripts without losses introduced by washing. Also for blood samples reagents have been developed that lyse the cells immediately and stabilizes the RNA for downstream applications. Comparison of direct lysis of blood samples with collection in traditional EDTA tubes reveals the impact of placing live cells in the very foreign EDTA medium that chelates (in particular) Mg2+ ions (Zhan et al). Divalent ions are common enzymatic co-factors and their removal has major impact on the transcript levels of many genes (Pazzagli et al, 2013). To preserve native expression levels in tissues samples they too have to be preserved, which for RNA analysis is preferably done by flash freezing (Kap et al, 2015). However, for routine samples morphological investigation is usually most important and those samples are preserved by formalin fixation and paraffin embedding (FFPE). This procedure does kill the cells and completely inhibits RNA metabolism, which preserves the native RNA levels; however, the procedure severely damages the nucleic acids, leading to very low and uncertain measurements that also may suffer from serious bias. Measured RNA levels may also be affected by transportation due to delays before the samples can be preserved and by storage. Usually storage itself, if done according to established procedures, does not impact sample quality, but the thawing of the sample does. When a sample is frozen, cells burst and nucleases get access to the nucleic acids. At low temperature (typically -80 ^{0}C) enzymatic activity is negligible, however, as the sample is thawed nuclease will digest. Therefore, freeze-thawing shall be avoided and frozen material should be processed whenever possible (i.e., frozen tissue is placed in the bead mill). Purified nucleic acids are preferably frozen in stabilizing solution (Tataa). Exposure of nucleic acids to radiation (UV or x-ray) or to reactive chemicals may also severely compromise the nucleic acids. Despite all precautions taken to preserve the native levels of the nucleic acids, their quality should be tested prior to analysis.

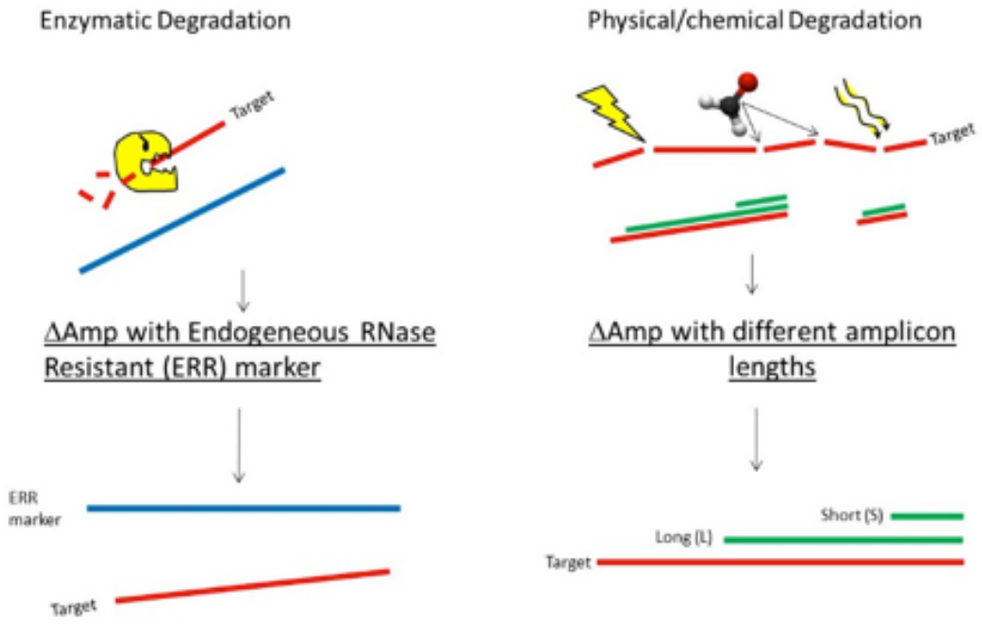

Figure 2. The Amp principle. An Endogenous RNase Resistant (ERR) marker is targeted with two assays that produce amplicons of different length. Comparison of the Cq's of the two different length amplicons reveals RNA integrity, while comparing the Cq's of the ERR and a reference gene (RG) reflects the degree of complete RNase degradation.

Currently most common test for RNA integrity is to analyze its length distribution using electrophoretic methods on microfluidic platforms such as the Bioanalyzer (Agilent) or Experion (Bio-Rad), the ScreenTape (Agilent) or capillary electrophoresis such as the Fragment Analyzer (Advanced Analytical) and QIAxcel (Qiagen). Bulk RNA is dominated by ribosomal RNAs (rRNA) that make up about 85% of the total amount. Most eukaryotes have two ribosomal subunits composed of four rRNA molecules. In human these are 5S (121 bases), 5.8S (156 bases), 18S (1869 bases) and 28S (5070 bases), where S is the Svedberg unit reflecting sedimentation, and they are readily separated electrophoretically. The 28S, 18S and 5.8 S rRNA molecules are encoded by a single transcriptional unit and therefore produced in equal amounts, resulting in a defined relation between their electropherogram peaks for fully intact material. Because of the much larger sizes of the 28S and 18S rRNAs they dominate the electropherogram and integral RNA is characterized by two distinct peaks in the electropherogram. Degradation of RNA is reflected by the broadening of the 28S and 18S rRNA peaks towards faster migrating molecules and a general increase of background due to accumulation of degradation fragments of various sizes. Analysis of the entire electropherogram determines a score that reflects RNA integrity. Different platforms deliver scores calibrated differently; the most common being the RNA Integrity Number, RIN, originally introduced by Agilent. RIN is a number between 1 and 10, where 10 reflects fully intact RNA and 1 totally degraded RNA. An analogues score is the RNA Quality Index (RQI) introduced by Bio-Rad, the RNA Quality Number (RQN) introduced by Advanced Analytical, RNA Integrity Number equivalent (RINe) introduced by Agilent, and the RNA Integrity Score (RIS) introduced by Qiagen. Although analyzing length distribution of RNA is today the far most common approach to evaluate RNA integrity, its relevance for gene expression analysis has occasionally been called into question. As outlined above the analysis reflects rather the integrity of ribosomal RNAs, which are quite different molecules from the

messenger RNAs and microRNAs typically studied. rRNAs have compact structures and are found in tight complexes with proteins, while mRNAs have 3' A-tail and 5'-cap that protect from nucleolytic degradation by binding proteins. mRNAs may also harbor internal sequences that are cleaved by endonucleases. Indeed, model studies of RNA degradation show rRNAs are degraded at quite different rates from mRNAs (Sidova et al, 2015). rRNA expression is further not expected to be appreciably affected by changes in environmental conditions, which may have profound effect on the expression of mRNAs (Rainen et al, 2002). The use of RIN to assess samples for miRNA analysis is even more questionable because of their very short length and empirical data supporting such correlation is limited (Ibberson et al, 2009). The impact of the chemical and structural differences between rRNAs and mRNA/miRNAs may further depend on the actual degradation mechanism. Still another limitation of electrophoretic assessment of RNA integrity is the precision when assessing samples of poor RNA quality. While high RIN/RQI values tend to be reproducible and reliable, repeated analyses of poor quality samples often gives highly varying values probably due to difficulties in modelling the low level signals and background. Our experience is measurements of RIN < 5 tend to be uncertain making conclusions ambiguous. This is a major limitation of the electrophoretic method, since borderline samples tend to have RIN < 5 and more reliable assessment of mRNA quality of these samples is highly desirable to distinguish between those where mRNA levels can be measured with reasonable accuracy from those where results would be so uncertain and unreliable that analysis is only waste of time and money.

Because of the limitations of the electrophoretic approaches molecular methods have been proposed to assess RNA integrity. One approach is the 3'/5' assay, which exploits the proximity to the A-tail at the mRNA 3'-end (Nolan et al, 2006). Reverse transcribing the mRNA using oligo(T) primer most of the cDNA should contain the complement to the mRNA 3'-end sequence, while only full length cDNAs reverse transcribed from intact mRNAs will harbor the complement to the mRNA 5'-end sequence. Hence, by quantifying the presence of the 5'- and the 3'-end sequences of an mRNA using oligo(T) primed RT-qPCR, the ratio would be expected to indicate the fraction of full lengths mRNA and, hence, reflect mRNA integrity. Although the 3'/5' assay has been reported to work well in some model system (Die et al, 2012), it has not been particularly useful for biological samples. One complication is that degraded samples contain many DNA and RNA fragments that may prime the reverse transcription and the required specific oligo(T) priming specificity is lost.

A more recent molecular method called Amp employs amplicon length to assess mRNA integrity as well as induction (Björkman et

al, 2015). It is based on using paired qPCR assays that produce amplicons of different lengths from the same target (Figure 2). A comparison of ΔAmpL/S = CqL –CqS and RQI on RNA degraded by formalin exposure under controlled conditions is shown in Figure 3. For intact mRNA the Cqs of the short and long amplicons are virtually the same, while for fragmented RNA the Cq of the longer amplicon is increased relative to that of the shorter. ΔAmpL/S is very sensitive to the integrity of the mRNA and changes more smoothly than the RQI with formalin exposure.

mRNA that is exposed to RNases remains predominantly intact as long it is protected from degradation at its 3'- and 5'-ends and when protection is lost the entire mRNA is rapidly degraded. Δ AmpL/S, comparing amplicons of different lengths is therefore not particularly sensitive to nuclease degradation. However, extensive screening has revealed some RNAs are inert to RNases. By instead comparing the Cq of such an inert RNA, referred to as Endogenous RNA Resistant (ERR) with the Cq of a reference gene (RG) the ΔAmpERR= CqRG–CqERR reflects loss of mRNA due to RNase degradation. Figure 4 compares RQI and Δ AmpERR for RNA degraded by RNases in tissue samples under controlled conditions. At modest degradation down to about RQI/RIN of 4 RQI/RIN and ΔAmpERR are congruent suggesting both approaches reasonably well assess the mRNA quality of the sample, even though the electrophoretic approach senses rRNA rather than mRNA. More extensive RNase degradation of mRNA is, however, sensed only by the ΔAmpERR.

Radiation can also damage nucleic acids. UV radiation introduced thymidine dimers that cannot be transcribed or reverse transcribed. The modification, however, does not break the mRNA. It is therefore not sensed at all by electrophoresis and RIN/RQI assessment, while it is sensitively detected with ΔAmpL/S. (Die et al, 2012)

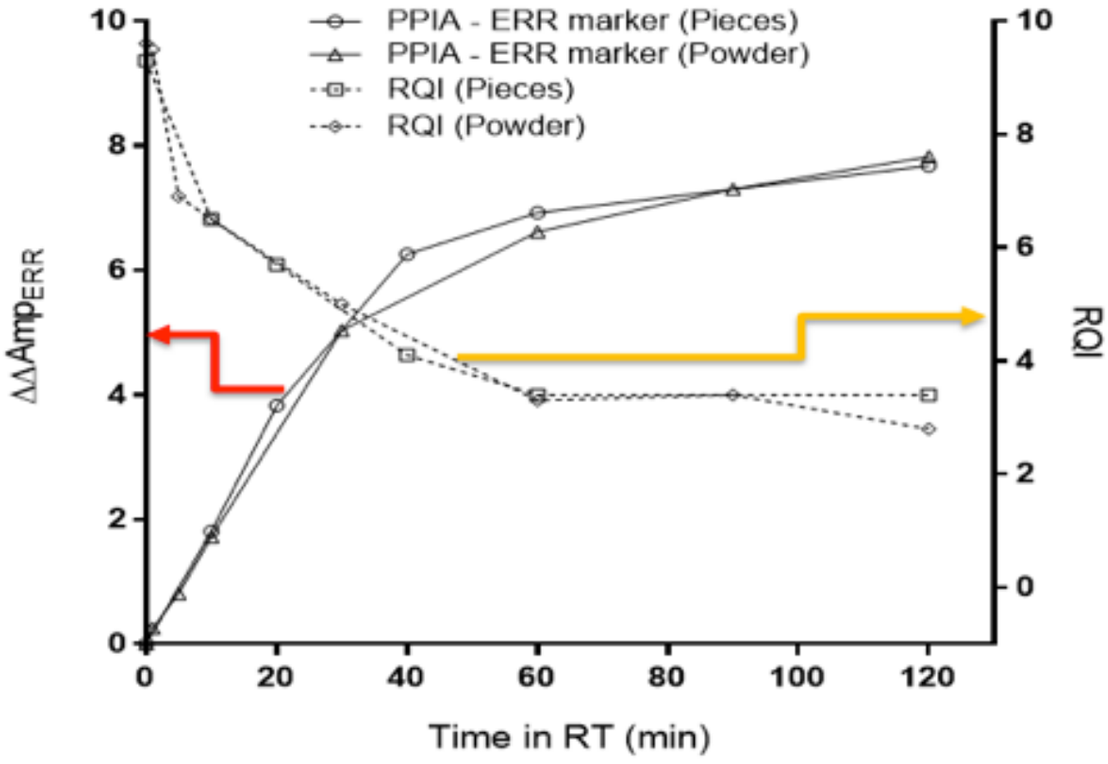

Figure 3. ΔAmpL/S = CqL –CqS normalised also to time = 0 (and therefore presented as ΔΔAmpL/S) and RQI measured on samples exposed to formalin for different lengths of time.

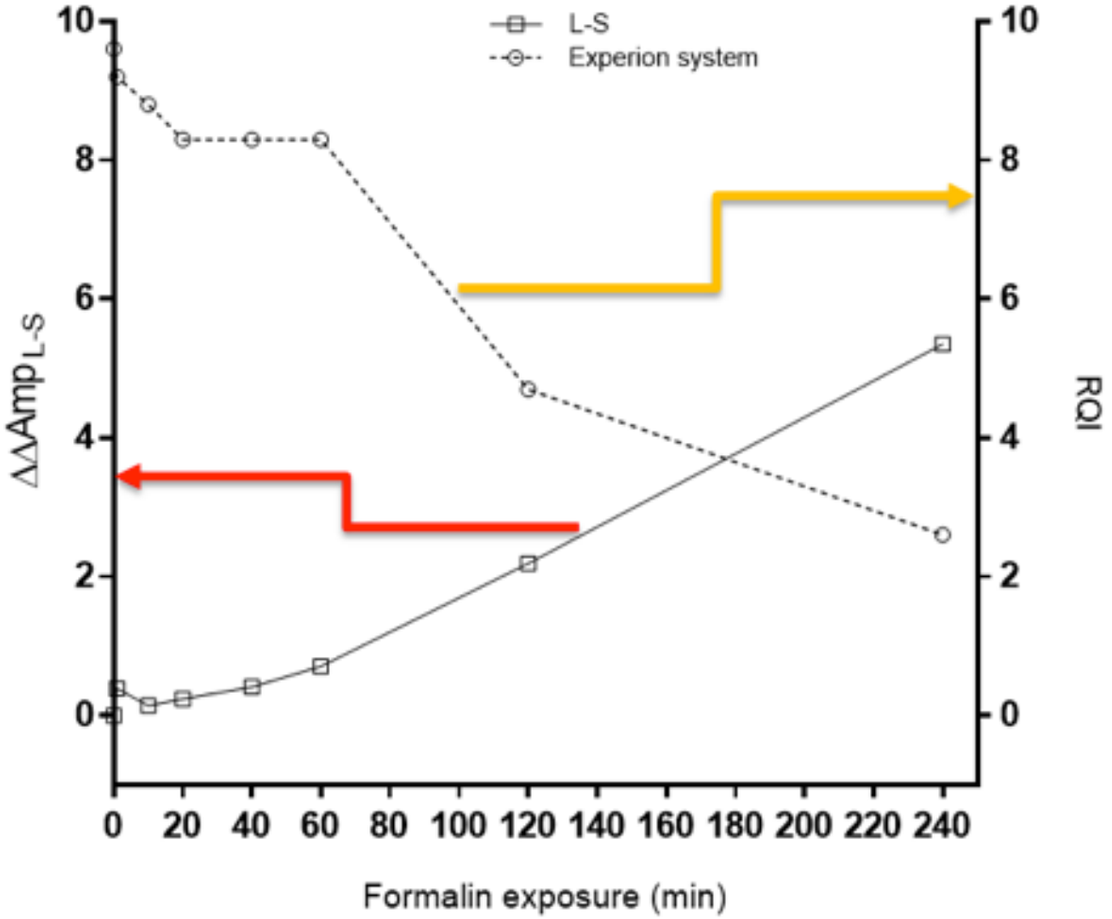

Figure 4. ΔAmpERR= CqRG–CqERR normalised also to time = 0 (and therefore presented as ΔΔ AmpERR) and RQI measured on tissue samples (liver piece or powder) exposed to room temperature for different lengths of time.

Testing for inhibition

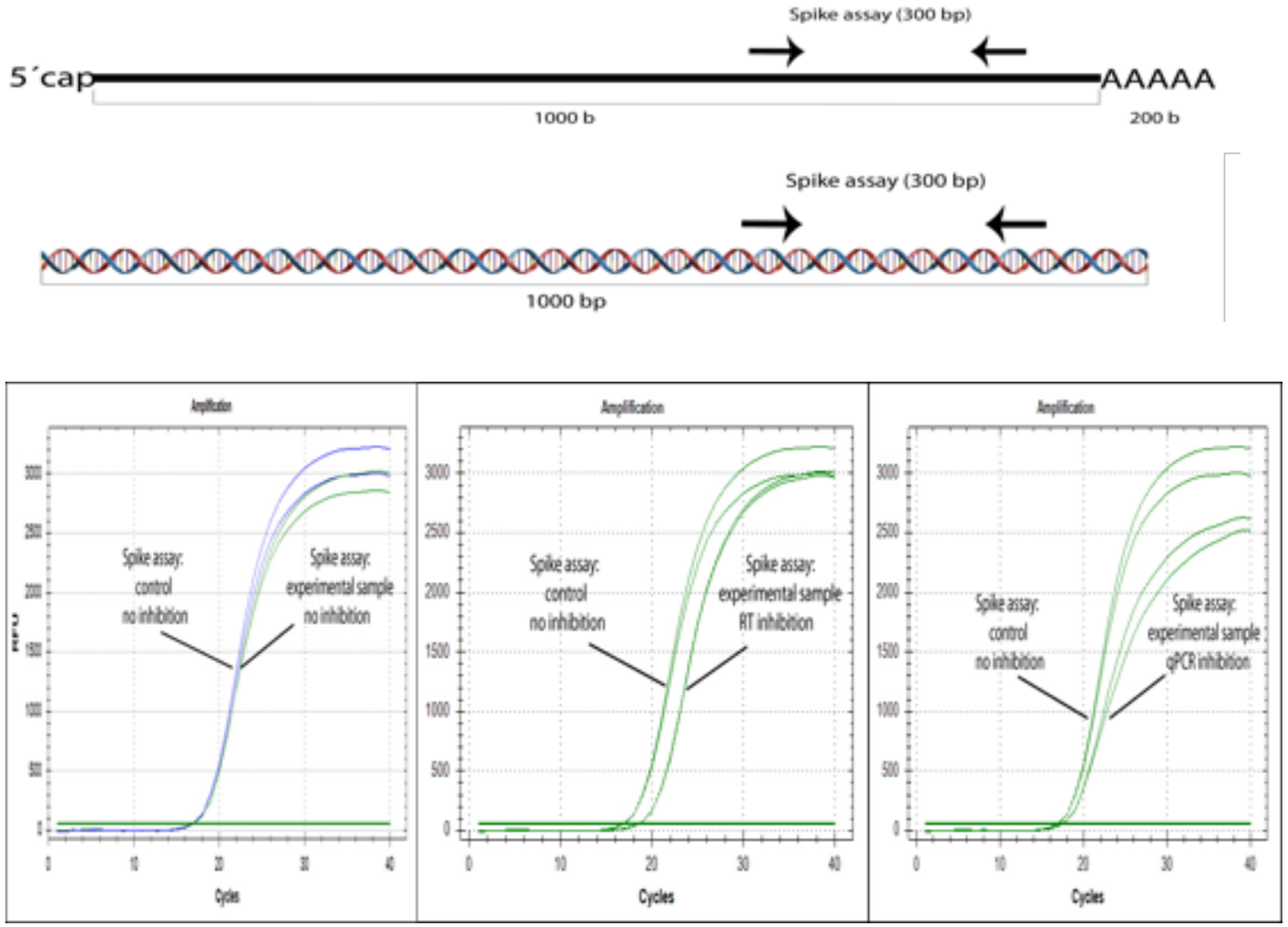

Figure 5. Top: design of RNA and DNA spikes used at TATAA Biocenter. Bottom: Comparison of RT-qPCR responses of the RNA spike in a test and control sample reflecting no inhibition (left), RT-inhibition (middle), and PCR inhibition (right) in the test sample.

Many biological samples contain components that severely inhibit RT and PCR, such as hemoglobin, myoglobin, bile salts, humic acids, urea, and collagen (Radström et al, 2004), and require extensive purification. However, extensive purification leads to losses and is expensive and many workflows has to compromise on purity. Furthermore, many of the reagents used to purify nucleic acids, such us phenol, chloroform, guanidinium thiocyanate, guanidine HCl, EDTA, DMSO, SDS, Qiazol, Trizol and RNAlater, are most potent RT and PCR inhibitors themselves and will severely inhibit downstream molecular reactions if carried over. Since inhibition has profound effect on the quantification by RT-qPCR it is essential to have it under control. Note, it is not necessary to completely remove inhibition; in fact it may not be possible, but then all samples compared should be inhibited to the same extent and inhibition should be modest at most. In any sample that is inhibited differently from the rest RNA levels will be erroneously quantified. Inhibition can be tested for using exogenous controls, also known as spikes. The spike can be either an RNA or a DNA molecule, depending on the target. RNA spikes are preferred when controlling mRNA expression analyses, while DNA spikes are preferred for DNA targets. The spikes are typically a few thousand bases/base-pairs alien sequence so the assays do not cross react with any known targets. The RNA spike is further modified with a 5'-cap and a 3'-A-tail to mimic the properties of mRNA (Figure 5). The spike should be added ahead of the step tested for inhibition. When testing the workflow the spike should be added as early as possible to the reaction, since it will reflect the efficacy of the analysis from its addition. Although not practical in the majority of workflows, ultimate use of a spike is to microinject it into the cells thus controlling for the entire process of analysis. Care must be taken, though, not to add the spike into a matrix that would degrade it, while the endogenous target is protected, for example, by being encapsulate as viruses are in blood. We use spikes extensively when developing new workflows and analysis methods. One can then add different spikes at different steps of the protocol to test their specific yields and identify steps that are suboptimal. Spikes can also be used to compare procedures, protocols and kits. Note, though, that the spike reflects yield, while for accurate analysis reproducibility is the key factor. Reproducibility of a workflow is optimized by performing a fully nested pilot study to identify the most confounding steps (Tichopad et al, 2009). We use spikes in routine when we have reason to expect or fear that some samples can be exceedingly inhibited. In parallel to the test sample the spike is added to a control sample that should not be inhibited. The samples are treated identically and their responses are compared. If the responses are the same the test sample is not inhibited, while a difference reflects inhibition. RT as well as PCR inhibition increases Cq, while only PCR inhibition affects the shape of the qPCR response curve, making it possible to identify the inhibited step (Nolan et al, 2006) (Figure 5).

Section 4

Interplate Calibration

Large profiling studies can often not be fitted into a single plate and multiple qPCR runs are needed to analyze all the samples for all the markers. The data shall then be merged into a common analysis. A complication doing this is that qPCR instruments set base line as well as threshold level for readout of the Cq values separately for each run, which may introduce bias between the runs. There is no bias if an “All Samples Layout” or an “All Genes Layout” is used, since the differential offset between runs cancels, but may be substantial if the layout is mixed. A mixed layout requires using interplate calibrator to account for the bias. An interplate calibrator (IPC) is a PCR common to all the plates that is used to measure the bias: any variation in its Cq values across plates reflects interplate variation and is the compensation that should be made. The IPC should be a highly robust reaction, which is obtained using purified template at a concentration that produces rather low Cq’s where reproducibility is high (for conventional qPCR cycles the Cq of the IPC should typically be in the range 20-25 cycles) that is measured with a highly efficient assay. Furthermore, the IPC should be run in replicates to reduce random noise. When the reactions can be plated using either “All Samples Layout” or “All Genes Layout” IPC is not necessary. However, good practice is to include it anyway. It does not hurt, but if the study later is expanded with additional samples as well as additional assays, the IPC will be needed for the merging of the new data. It is quite straight forward to make your own IPC, just make sure it is stable at least for the duration of the study. Alternatively, IPCs are

available from vendors, who provide IPC in aliquots that can be stored using one at a time to make sure all the reagents are from the same batch (Tataa).

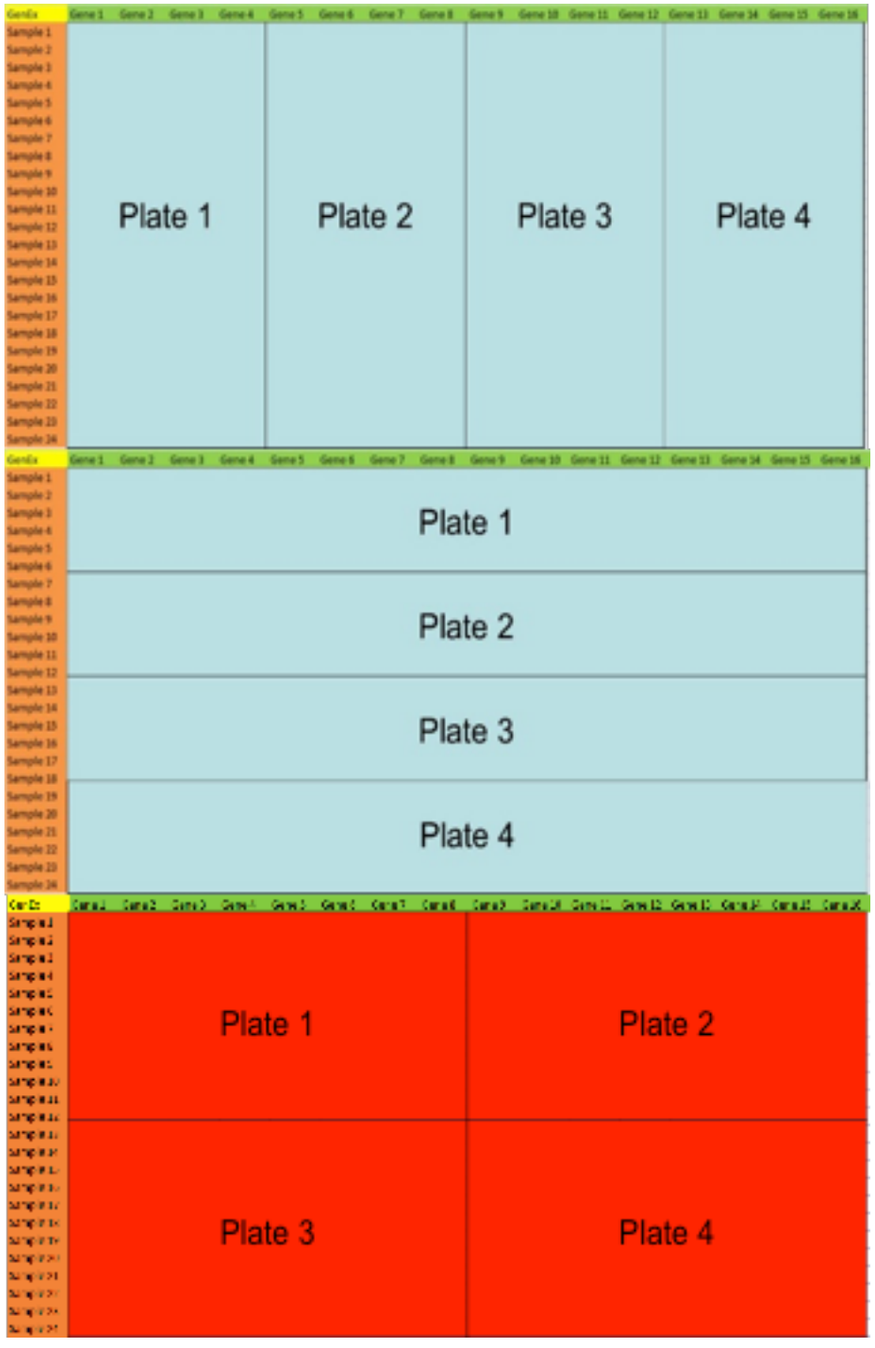

Figure 6. Layout for multiplate measurements illustrated for the case with24 samples analyzed for 16 markers. Top: "All Samples Layout", Middle: "All Genes Layout", Bottom: an example of a mixed layout.

Section 5

Genomic Background

The primers used to amplify cDNA in RT-qPCR may also amplify any residual genomic DNA (gDNA) present. Although many protocols include DNase treatment, it does not remove the DNA completely because too harsh treatment is precluded by the limited specificity of DNases for DNA. Assays for cDNA are, whenever possible, designed to span introns alternatively to cross exon-exon junctions to suppress amplification of genomic copy of the gene. If the intron spanned is reasonably long (at least over 1000 base-pairs) the amplicon that would be produced from the genomic copy will be too long to be amplified efficiently when using limited extension time in the PCR. This design strategy usually works well. However, not all genes have introns and genomic amplification of those cannot be avoided. Furthermore, a significant fraction of genes have pseudogenes (in human about 15 %) and about 50% of those lack introns as they were produced by retrotransposition of the transcript. These pseudogenes will be amplified also by intron spanning primers. Pseudogenes are particularly common among housekeeping genes and the number of pseudogenes can be quite large: for example, in human 68 pseudogenes for GAPDH have been reported (Pei et al, 2012). Hence, genomic DNA background can be substantial even when using intron spanning primers.

Traditionally, genomic DNA background is measured by performing a parallel set of control reactions, where reverse transcriptase is left out. These RT- controls

produce no cDNA and, hence, measure only the genomic DNA background. The difference in Cq's between the RT- and RT+ reactions should be at least five cycles to consider the gDNA background negligible. This criteria has been used for years and is considered robust. However, the approach is costly, since all reactions, RT as well as qPCR, has to be run twice. Recently, Henrik Laurell developed a much more cost efficient approach called ValidPrime (Laurell et al, 2012). ValidPrime is a highly optimized PCR assay targeting a non-transcribed conserved locus present in exactly one copy per haploid genome. Hence, ValidPrime measures the gDNA background also in the presence of cDNA. Only ValidPrime is, however, not sufficient to correct for gDNA background in RT-qPCR, because it is not known how sensitive the assays used are for gDNA. Analyzing a gDNA standard with all the assays provides this information and correction is then possible:

$$Cq_{RNA}^{GOI} = -log_2(2^{-Cq_{RT+}^{GOI}} - 2^{-Cq_{RT-}^{GOI}})$$

Where

$$Cq_{RT-}^{GOI} = Cq_{Sample}^{ValidPrime} + (Cq_{gDNA}^{GOI} - Cq_{gDNA}^{ValidPrime})$$

The correction is exceedingly accurate and up to 50% gDNA can be accounted for.19 It also saves cost: analyzing m samples for the expression of n genes, traditional RT- correction requires m RT control reactions followed by mn control qPCRs, while with ValidPrime control RT reactions are not needed and m+n+1 control qPCRs are sufficient. ValidPrime kits are available for human and mouse as well as a general vertebrate assay (Tataa - ValidPrime). The human gDNA control included is calibrated against NIST SRM 2732 (NIST), allowing absolute quantification of the gDNA content. The kit also contains complimentary GenEx license for the analysis (MultId).

Original data	gene 1	gene 2	gene 3	gene 4	ValidPrime
sample 1	20.1	31.1	22.1	28.2	32.5
sample 2	20.5	31.2	22.5	28.9	33.2
sample 3	21	31.1	22.9	30.2	32.3
sample 4	23.1	31.8	22.5	32.3	34.2
sample 5	23.5	30.8	22.8	32	33.1
gDNA standard	25.8	26.9	26.7	26	27

Figure 7.Experimental RT-qPCR layout including correction for gDNA background with ValidPrime.

Section 6

Conclusions

Measuring an RT-qPCR signal with an assay targeting a transcript of interest is easy and the data generated often look good, but it is a different story if the signal truly reflects the amount of transcripts of the targeted gene that actually were present in the biological context. This has to be validated by performing relevant controls. We describe quality control measures to test for degradation of RNA, inhibition, genomic DNA background and we also provide means to compensate for interplate variation. These controls should be performed in addition to the optimization of the RT-qPCR protocol itself, which is described in the MIQE guidelines (Bustin et al, 2009). For inexperienced users and also for laboratories setting up these quality control measures for the first time hands-on training courses are available (Tataa).

Section 7

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Chapter 10

Choosing reference genes

Philip Zimmermann

Nebion

Section 1

Introduction

RT-qPCR data are frequently normalized using internal control genes expected to have a stable level of expression across all samples of an experiment. The purpose is to eliminate external sample biases such as variations in total RNA content, RNA stability, enzymatic efficiencies or sample loading. Such control genes are called “reference genes” as recommended by MIQE (Bustin et al., 2009), formerly often referred to as “housekeeping genes”. The choice of suitable reference genes is absolutely crucial in RT-qPCR gene expression quantification. Historically, most laboratories have chosen genes having a “housekeeping” function to normalize RT-qPCR data, with the assumption that their expression level is likely to be constant in their experiment.

Section 2

Problems related to the choice of reference genes

In the past few years, hundreds of studies have been published revealing that classically designated reference genes such as GAPD, ACTB, PPIA or B2M, fail to normalize well in certain contexts. This is primarily due to their inherent variability of expression in particular conditions. It has therefore been recommended to experimentally validate several reference genes in one's own experiment and to use only those having a proven higher stability of expression (e.g. as selected using geNorm (Vandesompele et al., 2002)).

A further problem with most classically designated reference genes like GAPD is that their transcripts are extremely abundant as compared to the transcriptome. Although this may theoretically not be an issue in RT-qPCR as long as the normalization is done within the linear range of amplification for each gene, this constraint is often not verified and biases may occur when normalizing weakly expressed genes with very strongly expressed reference genes. It is therefore recommended to include additional reference genes expressed in the lower or medium range of overall transcript abundance.

Section 3

Choosing appropriate reference genes

To address the main problems associated with classically designated reference genes, several laboratories have made use of the large collections of microarray data to identify novel reference genes having stable expression in selected biological contexts, such as a cancer type or a tissue type (Czechowski et al., 2005; Saviozzi et al., 2006; Stamova et al., 2009; Gur-Dedeoglu et al., 2009; Popovici et al., 2009; Lee et al., 2007). Experimental validation in those contexts proved these novel genes to perform better in normalizing RT-qPCR data than commonly used reference genes. The use of microarray data has therefore been proven to be a valid and useful approach for identifying condition-specific reference genes. Using a very large set of microarray data, Hruz et al. (Vandesompele et al., 2002) demonstrated that

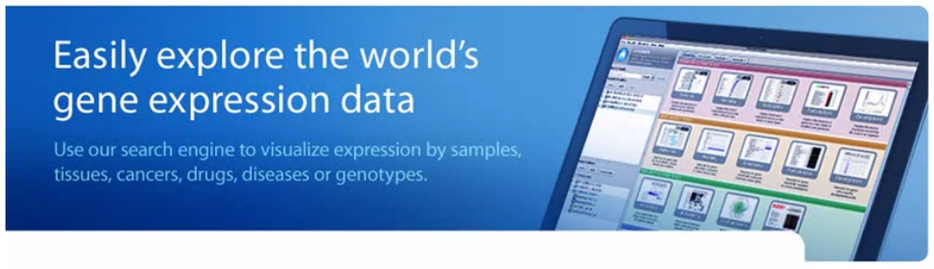
a) no gene is universally stable across all conditions, and

b) for every biological context, a distinct set of genes exists having the least variance of expression.

RefGenes (Hruz et al., 2011; Refgenes) is a free online tool developed specifically for this purpose. It allows users to choose a tissue or condition of interest, to identify genes being stable in the chosen condition, and to verify in-silico their expression stability across a wide compendium of perturbations. Once the identification and prioritization of reference genes based on microarray expression

data is done, the identified candidate reference genes must be experimentally validated in one's own experiment. In general, it is not recommended to use less than three reference genes for normalization of RT-qPCR data.

Short videos on how to use RefGenes are provided just below.



Introduction to **GENEVESTIGATOR**

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Chapter 11

The importance of efficiency-correction in qPCR

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Summary

The Y-axis title of a graph showing results of a qPCR experiment most often indicates that the axis represents the fold difference of the normalized expression of the target gene between the experimental and control conditions. However, in most cases the presented data are Cq, Δ Cq or ΔΔCq values. Nowadays the '2' and the 'minus sign' that should be there to turn a ΔΔCq into a fold-difference (Livak and Schmittgen 2001) have disappeared almost completely from publications. This tendency to avoid formulas is turning quantitative PCR into a field of meaningless numbers. For examples to illustrate this phenomenon, just google ddCq.

Although this situation gives the impression that ΔΔCq needs no explanation for the users of qPCR, the opposite is true. The reviewer and the reader are satisfied by the presence of significant effects illustrated by asterisks in the graphs. The reported results are significant and that is what is important. In the best-case scenario, the authors, reviewer and readers have done some mental arithmetic and interpreted the data correctly. Although then they must have assumed that the qPCR assays reported in the experiment are all equal and all are 100% efficient.

Section 2

Efficiency-corrected relative quantification

The above mentioned Δ Cq and ΔΔCq originate from the equations to calculate the fold-differences between a target gene and a reference gene ($2^{-\Delta Cq}$) and later on in a certain condition (e.g. disease) and control ($2^{-\Delta\Delta Cq}$) (Livak and Schmittgen 2001). Although these equations were introduced to simplify the interpretation of qPCR results, the presentation of only the ΔΔCq values in papers is now complicating the interpretation of reported results. Moreover, using these equations ignores differences in PCR efficiency between target genes and reference genes which can lead to severe bias in the interpretation of the reported data. Since the introduction of qPCR it was highlighted by experts in the field and fixed in the MIQE guidelines that ΔCq and ΔΔCq cannot be applied when there is a difference in amplification efficiency of the target and reference genes being compared (Bustin et al. 2009). Since this was and is almost always the case, a new equation was published, incorporating the difference in Cq values and the amplification efficiencies of target and reference leading to efficiency-corrected relative quantification:

$$Fold\ change = \frac{(E_{target})^{\Delta\ Cq_{target}}\ (control - treatment)}{(E_{ref})^{\Delta\ Cq_{ref}}\ (control - treatment)}$$ [Eq. 1] (Pfaffl 2001)

With the assumption that the PCR efficiency of the target gene and the reference gene are both 2, and a rearrangement of the Cq values in the exponent, this equation ‘simplifies’ to

$$Fold\ change = 2^{-((C_{q,T,Tr}-C_{q,R,Tr})-(C_{q,T,\lambda}-C_{q,R,\lambda})} \quad \text{[Eq. 2]}$$

which is the full write-out of the $2^{-\Delta\Delta Cq}$ equation (Livak and Schmittgen 2001). These equations can easily be applied when only one reference gene and no biological replicates are used; the average Cq of technical replicates per sample can then be inserted in the equation. When the experiment includes biological replicates of treated and control samples the application of Eq. 1 and 2 becomes more difficult. This is because the control and treatment samples are unrelated and the researcher has to use error propagation or bootstrapping to determine the confidence interval of the resulting and reported fold-change (Tellinghuisen and Spiess 2014; REST software manual). Similarly, the application of more than one reference gene necessitates more complicated calculations (Hellemans et al. 2007).

To easily handle biological replicates and multiple reference genes one can use a sequence of calculations that goes back to the original kinetic equation of the PCR reaction

$$N_C = N_0 . E^C \quad \text{[Eq. 3]}$$

with N0 as the unknown starting concentration or target quantity, Nc as the amount of product after C cycles and E as the amplification efficiency. By definition Cq is the number of cycles needed to reach the quantification threshold Nq. Therefore, the target quantity N0 can be directly derived from inversion of this kinetic equation at Nq:

$$N_0 = N_q / E^{Cq} \quad \text{[Eq. 4] (Ruijter et al. 2009)}$$

Eq. 4 shows that when the quantification threshold is set and the Cq is determined for a reaction amplifying the target gene with efficiency E, the target quantity of this gene in the reaction can be calculated. The normalized expression of the target per tissue sample can then be calculated from the target quantities (averaged over the technical replicates) of the target and the references:

$$norm.expr. = N_{0.target} / geomean(N_{0.references}) \quad \text{[Eq. 5]}$$

where the denominator is the geometric mean of the target quantities of the references (Hellemans et al. 2007). The technical variation of the target quantities and normalized expression in qPCR experiments is relatively small (Kitchen, Kubista, and Tichopad 2010) and mainly serves as a quality control.

The normalized expression values per tissue sample (Eq. 5) can be averaged over biological replicates in the different experimental treatment groups. These means and their variation are the used in statistically comparisons between experimental conditions in accordance with the experimental design. Note that it is not required to define a control condition

Section 3

Bias caused by assuming that all PCR reactions are 100% efficient

The effect of the simplifying assumption that all PCR assays amplify 100% efficient (Eq. 2) will be illustrated with an example dataset. Four target genes and a reference gene are measured in an experiment with three treatments and a control condition (Table 1). The target genes all have the same expression level but show a four-fold increase between the control and each of the three treatments; the expression level of the reference gene is by definition the same in all conditions. The reference and the targets are amplified with different PCR efficiencies and the Cq values that would be observed were determined (Table 1 and Supplemental Excel file, sheet 1).

		control	treatment_1	treatment_2	treatment_3
	all targets	10	40	160	640
	reference	160	160	160	160
	PCR efficiency	Cq	Cq	Cq	Cq
target_4	1.70	26.0	23.4	20.8	18.2
target_3	1.80	23.5	21.1	18.8	16.4
target_2	1.90	21.5	19.4	17.2	15.0
target_1	2.00	19.9	17.9	15.9	13.9
reference	1.85	18.0	18.0	18.0	18.0

Table 1: Input variables for the illustration of the effect of ignoring the target-specific PCR efficiency values. Results of calculations with these numbers are shown in Fig. 1. Other expression scenarios can be tested in the Supplemental Excel file, sheet 1.

These Cq values and the efficiency values per target were used in Eq. 4 and 5 to calculate the fold-change (Fig 1). What is the effect when this difference is ignored and all assays are assumed to be 100% efficient. For target 1, amplified with an efficiency of 2, the result is the correct fold difference in each of the treatments, regardless whether Eq. 2 or the combination of Eqs. 4 and 5 is used for the calculations (Fig. 1). For treatment 1, with only a 4-fold difference compared to the control, the use of Eq. 2 results in only slightly deviating values, even when the efficiency value of the target is far below 2. However, with increasing effect of the treatment and decreasing PCR efficiency of the targets the bias caused by the use of Eq. 2 becomes increasingly clear (Fig. 1).

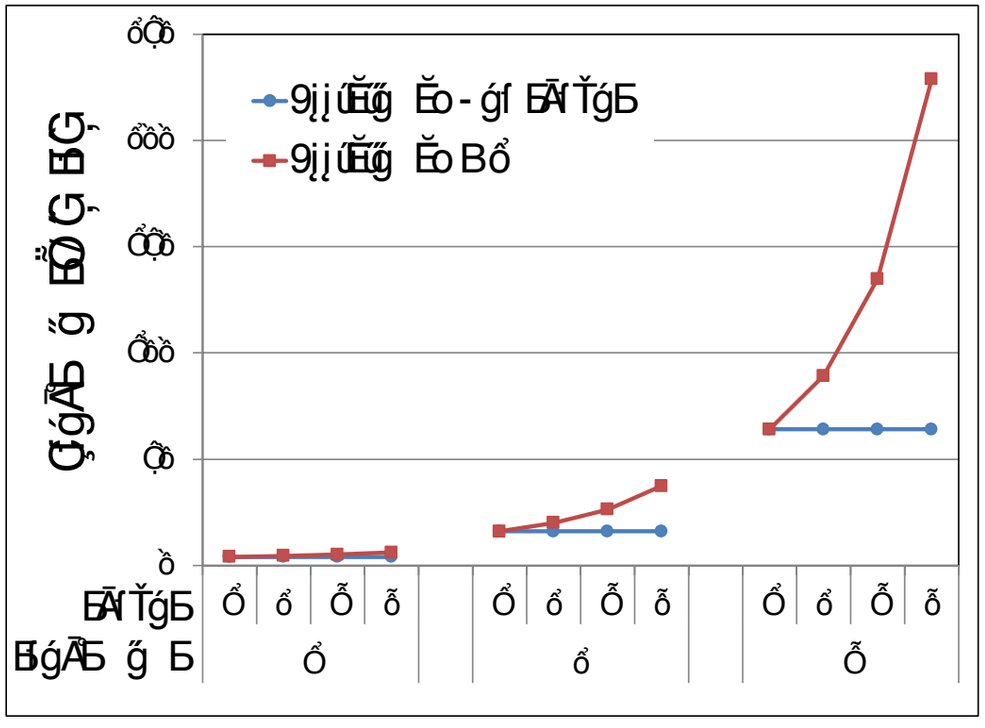


Figure 1: Illustration of how ignoring the target specific PCR efficiency values leads to wrong conclusions

Table 1 shows that for all targets the starting copy numbers are the same, increasing 4-fold between control and subsequent treatments. The different amplification efficiencies of the targets make that for each target and condition another number of cycles (Cq) is needed to reach the quantification threshold. The fold-difference of the normalized gene expression between treatment and control conditions is 4, 16 and 64 for all targets per treatment (blue lines, calculated with the efficiency per target and the Cq values in the table and [Eq. 4 and 5]). When all efficiency values are assumed to be 2, the reported fold-difference between treatment and control (red lines, calculated with the Cq values in the table and [Eq. 2]) increases with decreasing PCR efficiency value and increasing true fold difference. Other expression scenarios can be tested in the Supplemental Excel file, sheet 1.

Given the sensitivity of qPCR, with enough biological replicates, the use of the ΔΔ Cq equation will lead to the conclusion that the targets are differentially expressed in treatments 2 and 3. This obviously wrong conclusion illustrates why the actual PCR efficiencies have to be included in the calculation of qPCR results (Bustin et al. 2009). Variations of the above effect can be studied with the simulation in the Supplemental Excel file (sheet 1). See also https://www.qbaseplus.com/knowledge/blog.

But why are the PCR efficiencies of the targets and references then commonly ignored and implicitly considered to be 2? Probably because most researchers, reviewers and readers think that in relative quantification the efficiency differences disappear from the calculation. The example shows that this is not the case. Therefore, we strongly recommend to use the efficiency-corrected relative quantification equation [Eq. 1] (Pfaffl, Horgan, and Dempfle 2002; REST software) or the efficiency-corrected starting concentrations (N0; Eqs. 4). The latter are calculated by the LinRegPCR program for analysis of the amplification curves (Ruijter et al. 2009). Both approaches will lead to highly similar results. Differences between the two approaches are caused by the PCR efficiency used in the calculations and the handling of the results of replicate measurements of control and treatment groups; averaging of Cq values in the case of Pfaffl, averaging of N0 in the case of Ruijter. The PCR efficiency will be discussed in the following paragraphs.

PCR efficiency derived from the standard curve

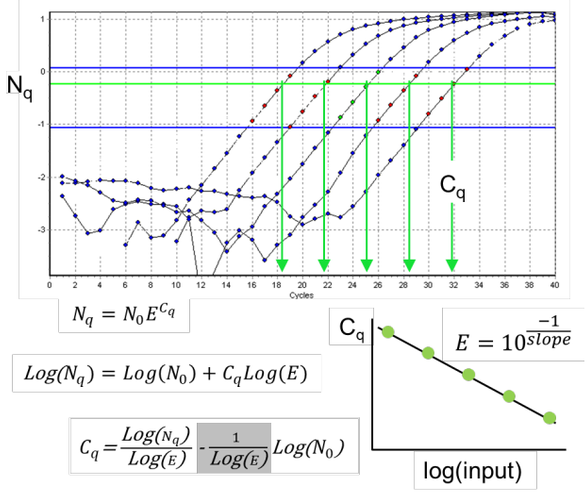


Fig 2: Illustration of the standard curve derived PCR efficiency

$$N_q = N_0 . E^{Cq}$$

$$Log_{(N_q)} = Log_{(N_0)} + C_q . Log_{(E)}$$

$$C_q = \frac{Log_{(N_q)}}{Log_{(E)}} - \frac{1}{Log_{(E)}} Log_{(N_0)}$$

$$E = 10^{\frac{-1}{slope}}$$

A PCR run of a 10-fold dilution series results in a series of parallel amplification curves. Only one observation per dilution is shown but in practice, standard curves have to be measured with technical replicates. After setting the quantification threshold (horizontal green line, Nq), the Cq values can be determined (vertical green arrows). A graph of the Cq

To apply efficiency-corrected quantification, the amplification efficiency per amplicon or qPCR assay has to be determined. The most common approach is to calculate the PCR efficiency from the slope of a so-called standard curve (Fig. 2). The standard curve is constructed after qPCR measurement of samples in a dilution series. The observed Cq values are then plotted against the 10log of the known input concentration or the 10log dilution factor when the target concentration in the undiluted material is not known. The equation of the regression line fitted to the data points is then

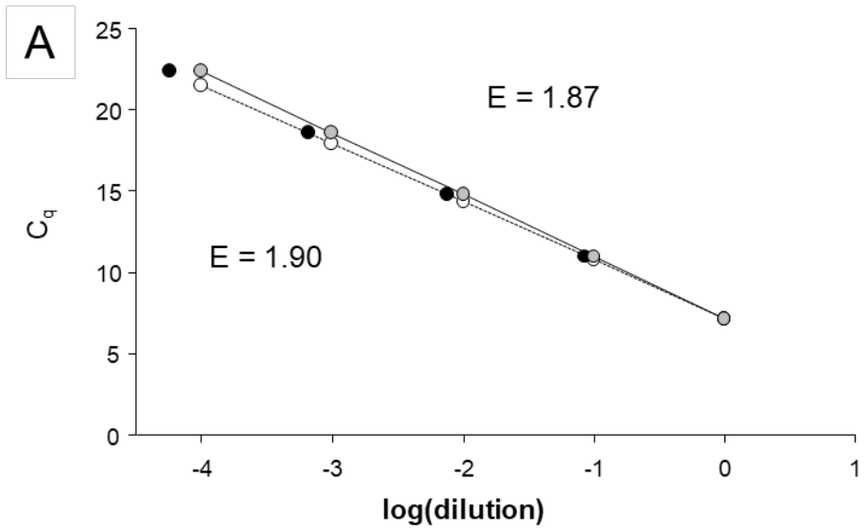
$$C_q = \frac{log(N_q)}{log(E)} - \frac{1}{log(E)} log(input) \qquad \text{[Eq. 6]}$$

From the slope of this regression line (-1/log(E)) the efficiency can be calculated as

$$E = 10^{\frac{-1}{slope}} \qquad \text{[Eq. 7]}$$

The calculations to obtain this standard curve slope derived PCR efficiency are implemented in most qPCR systems and most commonly used in published papers. However, although this approach is theoretically valid, technical and biological

variations in the preparation of the standard curve can and will affect the resulting PCR efficiency. Each of these technical and biological variations is illustrated with a simulation in the Supplemental Excel file (sheets 2-4).



Effect of systematic pipetting error on the observed PCR efficiency

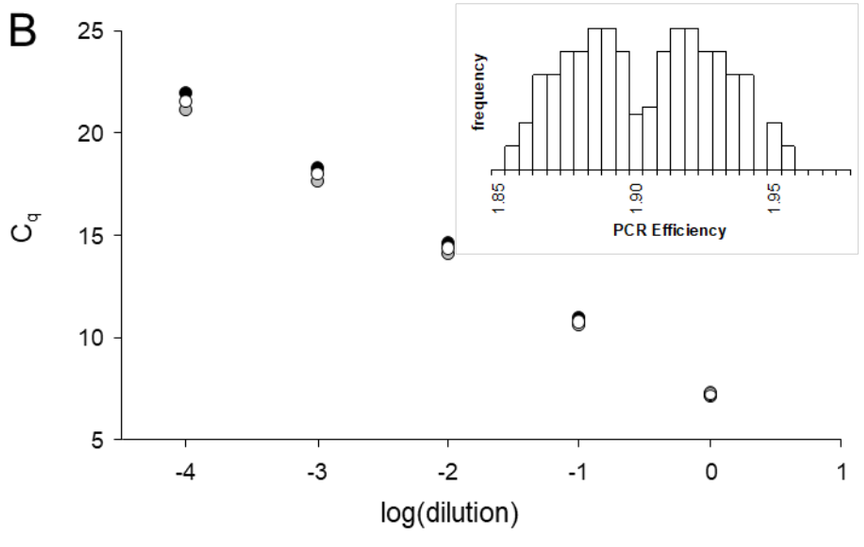
A systematic error in the dilution series, because of a wrongly calibrated pipet, will affect the standard curve. An unnoticed larger dilution step leads to less input in the reaction which leads to increasingly higher observed Cq values per dilution step. Plotted against the assumed correct log(input) these Cq values will give a steeper standard curve and thus a lower derived PCR efficiency (Fig. 3A). An unnoticed smaller dilution step has the opposite effect. This shows that the efficiency derived from a standard curve depends on the correct, and regular, calibration of the pipets. Because the samples that have to be measured are not diluted, the error in the PCR efficiency is not compensated for these 'unknown' samples. This means that the use of this standard curve as a calibration curve for absolute quantification will also give a wrong result even when the same pipet is used for processing the samples (Supplemental Excel file: sheet 2).

Fig 3 A, B and C: Illustration of how the standard curve can give a wrong efficiency value

Systematic or random pipetting errors make that for every planned dilution step there is a variation in starting concentration and observed Cq value. A: data points are simulated with a systematic pipetting error leading to 5% less input and 5% less dilution propagated through the dilution series. Because the user is unaware of this pipetting error, the observed Cq values (black spots) are plotted against the planned dilutions (grey spots) leading to a steep standard curve and a low derived PCR efficiency. White spots indicate the Cq values that would be observed without pipetting error. B: Random pipetting errors in every dilution step (grey spots: more input; black spots: less input) lead to a large range of possible standard curves. This leads to a range of observed PCR efficiency values (inset panel B). C: Dilution of a PCR inhibitor leads to a systematic increase of the PCR efficiency with each dilution step (inset panel C). The dilution thus causes increasingly lower Cq values. The standard curve is still a straight line but with a shallower slope (black spots) than the line that is found when the sample had a constant low efficiency (white spots) and thus results in a PCR efficiency value that is even higher than the efficiency in the maximally diluted reaction. Other expression scenarios can be tested in the Supplemental Excel file, sheet 2, 3 and 4.

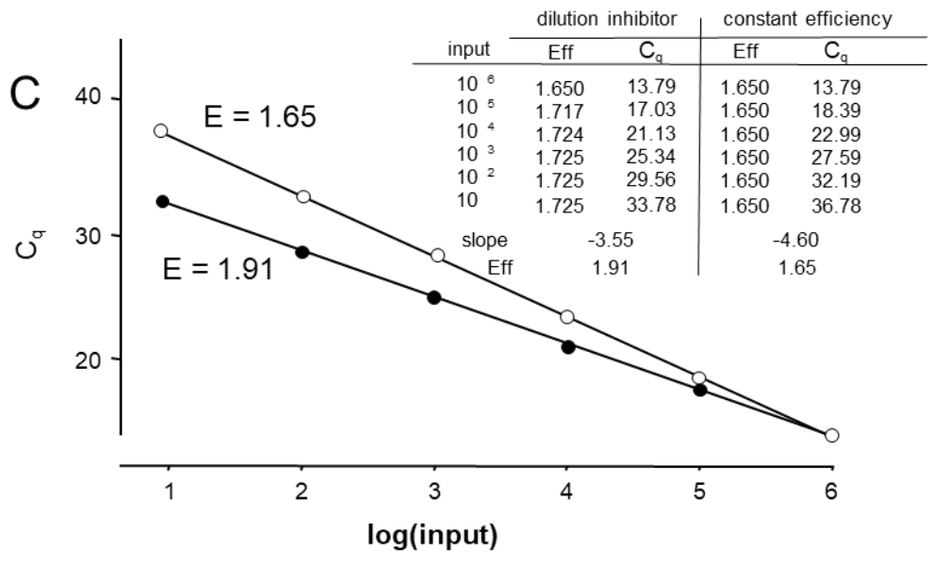
Effect of random pipetting error

Even with correctly functioning and calibrated pipets, random operator errors can and will occur. Because these pipetting errors are unknown to the user, the observed Cq values are again plotted against the assumed input. The input errors are thus treated as variation in the observed Cq values (Fig. 3B). A random pipetting error of 5% leads to less than a cycle difference in the Cq of the most diluted sample and will thus go unnoticed. Even a 15% pipetting error results in only 0.5 cycle error in Cq value (de Ronde et al. 2017; see also qPCR dPCR & NGS 2017 Event). However, when 5% pipetting errors occur randomly in the preparation of the dilution series in different experiments, they may result in a range of 10% in the PCR efficiency values derived from the standard curves in the different experiments (Ramakers et al. 2003; Ruijter et al. 2009) (Fig. 3B, inset; Supplemental Excel file: sheet 3).



Effect of a PCR inhibitor in the undiluted sample

The presence of a PCR inhibitor in the sample used to construct the standard curve leads to a low PCR efficiency in the undiluted sample experiments (Ramakers et al. 2003; Tichopad et al. 2003; Tichopad et al. 2010). However, dilution of the sample and thus dilution of the inhibitor will give a higher PCR efficiency after every dilution step (Fig. 3C, inset). This leads to progressively lower observed Cq values and thus to shallower standard curve. Note that the dilution of this inhibitor will go unnoticed because the standard curve is still a straight line (Fig. 3C). The derived PCR efficiency value is, however, even higher than the efficiency of the most diluted reaction (Fig. 3C, inset). This high standard curve derived PCR efficiency will lead to an under-estimation of the target quantity in the unknown samples.



	dilution inhibitor		constant efficiency	
input	Eff	C_q	Eff	C_q
10^6	1.650	13.79	1.650	13.79
10^5	1.717	17.03	1.650	18.39
10^4	1.724	21.13	1.650	22.99
10^3	1.725	25.34	1.650	27.59
10^2	1.725	29.56	1.650	32.19
10	1.725	33.78	1.650	36.78
slope	-3.55		-4.60	
Eff	1.91		1.65	

PCR efficiency derived from the amplification curves

Alternatively, the PCR efficiency can be derived from the individual amplification curves. Because PCR efficiency is defined as the fold-increase per cycle, it is possible to calculate the PCR efficiency from two consecutive cycles in the exponential phase of the reaction (Eq. 8).

$$E = \frac{F_{C+1} - F_b}{F_C - F_b} \qquad \text{[Eq. 8]}$$

This approach requires that the fluorescence baseline (Fb in Eq. 8) is subtracted from the observed fluorescence data. The 2 data points that are used in this calculation can be chosen as the two data points on both sides of the quantification threshold set by the user or as the two data points immediately preceding the second derivative maximum (SDM) that is calculated from the fit of an S-curve through the data points of the amplification curve (Spiess, Feig, and Ritz 2008). However, because only two points from the amplification curve are used, the variation of the calculated efficiency values can be large (Ruijter et al. 2013).

A better estimate of the PCR efficiency is obtained when the baseline-corrected fluorescence values are plotted on a logarithmic scale. The logarithmic form of the kinetic equation of PCR

$$log(N_c) = log(N_0) + c\ log(E) \qquad \text{[Eq. 9)}$$

shows that the data points observed in the exponential phase of the PCR are on a straight line when the fluorescence values are plotted on a logarithmic fluorescence scale. The slope of this line, log(E), is the logarithm of the PCR efficiency. The data points used to determine the PCR efficiency should be chosen in the exponential phase of the PCR and the PCR efficiency can then be calculated as:

$$E = 10^{slope} \qquad \text{[Eq. 10]}$$

Using a non-linear procedure to fit the kinetic equation for PCR (Eq. 3) to the data points in the exponential phase of the reaction (the baseline-corrected data points between the ground phase noise and the SDM) will give an estimate of the PCR efficiency without the need for log-transformation (Zhao and Fernald 2005).

The use of two or more data points in the exponential phase to determine the PCR efficiency is very sensitive to errors in the baseline correction. When the baseline value is estimated too high, the numerator and the denominator of Eq. 8 both include a negative constant which will always lead to a higher calculated efficiency. Similarly, a baseline that is estimated too low will lead to a low efficiency value. Fortunately, the shape of the amplification curve after baseline subtraction clearly shows incorrect baseline estimations. When the datapoints in the exponential phase, which are the datapoints preceding the cycle at which SDM is reached, are not on a straight line the baseline is set incorrectly (Ruijter et al. 2013).

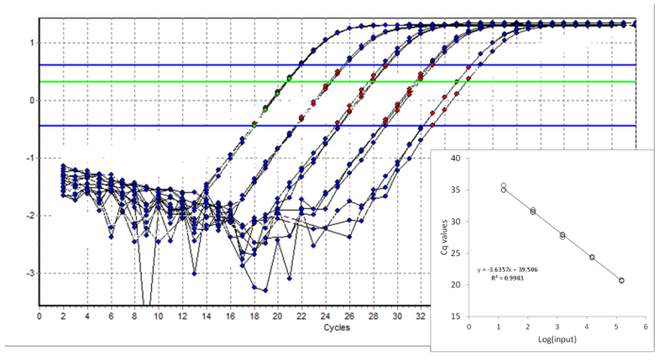
Which efficiency to use for calculating the target quantity, relative expression or fold-change

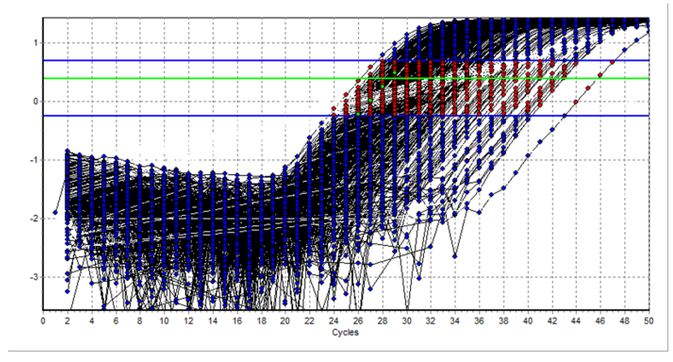
When the PCR efficiency is derived from a standard curve, a single efficiency value per amplicon or target is obtained and this efficiency will be used in the calculations for all reactions with that PCR assay. However, when the PCR efficiency is derived from the amplification curves, the researcher will obtain a PCR efficiency value for each individual reaction. Differences in observed PCR efficiency between reactions that amplify the same target have been assumed to reflect small, but real differences in reaction kinetics (Ramakers et al. 2003; Pfaffl 2001; Tichopad, Didier, and Pfaffl 2004 Tichopad et al. 2003). Therefore, several qPCR data analysis methods calculate the target quantity with those individual PCR efficiencies. However, the qPCR data analysis methods that had been made available to the public before mid-2012 were extensively evaluated. The results of this comparison of methods were presented at the 2013 qPCR event and showed that methods that applied a single PCR efficiency per target performed better than methods that applied a different PCR efficiency for each reaction (Ruijter et al. 2013; Tellinghuisen and Spiess 2014). To obtain such a single PCR efficiency per target, the efficiency values that are derived from individual amplification curves have to be averaged per target (Ruijter et al. 2009; Zhao and Fernald 2005). Experiments with a large number of reactions show that the observed PCR efficiencies per target are normally distributed with a small variation.

This illustrates that the differences between reactions are the result of random technical variations in the fluorescence measurement and baseline subtraction.

Comparison of both methods

A dataset from the above-mentioned comparison of qPCR data analysis methods (Ruijter et al. 2013) is used to illustrate and compare the methods to determine PCR efficiency values (Fig. 4). The dataset includes a dilution series (5 dilution steps each measured in triplicate: 15 reactions) and 366 patient samples (366 reactions). The PCR efficiency derived from the slope of the standard curve constructed from the Cq values observed in the dilution series is 1.88; the SE can be derived from the residual variation around the regression line). The average of the PCR efficiencies derived from the 366 amplification curves of the patient samples is 1.71 (with a SE of 0.004) showing that some systematic technical error was introduced in the dilution series. Moreover, the average PCR efficiency derived from the amplification curves of the 15 reactions of the dilution series was 1.84 (SE: 0.004). The difference between this value and the value found for the patient samples can be explained by the fact that the dilution series was prepared by diluting a plasmid containing the target whereas in the patient samples the target is present in the cDNA (Fig. 4).





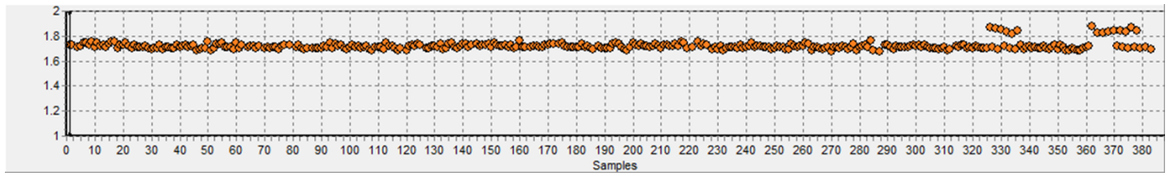


Fig 4: How to chose between different efficiency values

The dataset includes a dilution series (5 dilution steps measured in triplicate: 15 reactions, top panel) and 366 patient samples (366 reactions, bottom panel). The PCR efficiency derived from the slope of the standard curve constructed from the dilution series is 1.88 (top panel, inset). The average of the PCR efficiencies derived from the 366 amplification curves of the patient samples is 1.71, whereas the average PCR efficiency derived from the amplification curves of the 15 reactions of the dilution series was 1.84 (middle panel).

Example of the use of assay specific efficiencies to calculate target quantities

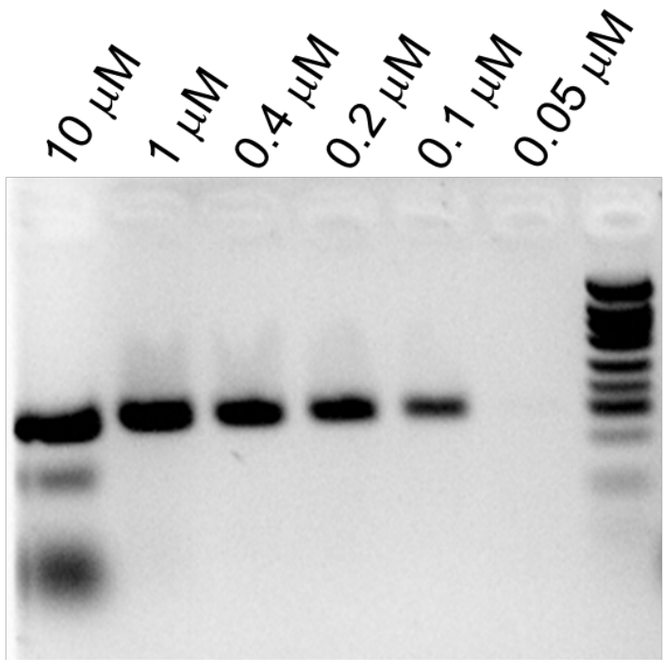


Fig 5A: How to chose between different efficiency values

Illustration of the use of assay specific PCR efficiency values. The electrophoresis gel shows that no product is amplified in the lowest primer concentration and that there is extensive artefact amplification for the highest primer concentration.

To investigate early claims that the PCR efficiency depends on either the target concentration or the primer concentration an experiment was done in which a dilution series of cDNA was combined with a range of primer concentrations. This experiment was presented at the qPCR 2011 Event The amplified target was ZNF248 (primer sequences,

FW: GAGCCCTGGATATTAGAAAAAGGAT,

REV: CACGTCATCAACTTTCCATTTCC).

The highest cDNA concentration was 6.5 ng, from which a 4-step, 5-times dilution series was prepared. The primer concentrations were 10, 1, 0.4, 0.2, 0.1 and 0.05 µM. Gel electrophoresis after the PCR run showed no product for the lowest primer concentration and extensive artefact amplification for the highest (Fig. 5A). These two extreme primer concentrations were excluded from further data analysis. Comparison of the observed Cq values showed that these significantly increased for lower cDNA input, as expected, but also for higher primer concentration (Fig. 5B). PCR efficiency values derived from the individual amplification curves showed a significant decrease with low primer concentration but no effect of cDNA input (Fig. 5C). This allowed, and required, the use of the average PCR efficiency per primer concentration for the calculation of the target quantities (N0) per reaction using Eq. 4, with the Cq value

per reaction. The resulting target quantities per cDNA input and primer concentration were not significantly different from the values expected in a 5-times dilution series (Fig. 5D).

This example shows that, because the four different primer concentrations resulted in different PCR efficiencies, the primer concentrations should be considered different ‘assays’, each resulting in different Cq values for the same diluted samples. With a PCR efficiency per ‘assay’ the effect of primer concentration on Cq was compensated resulting in the correct target quantity, despite the different, and for one ‘assay’ very low, PCR efficiency.

The experiment shows that the cDNA, or target, concentration does not affect the amplification efficiency of a qPCR assay. However, primer concentration does affect the PCR efficiency when the primer concentration is too low; there was no difference in PCR efficiency between the 1 and 0.4 µM primers (Fig. 5C) which shows the robustness of qPCR.

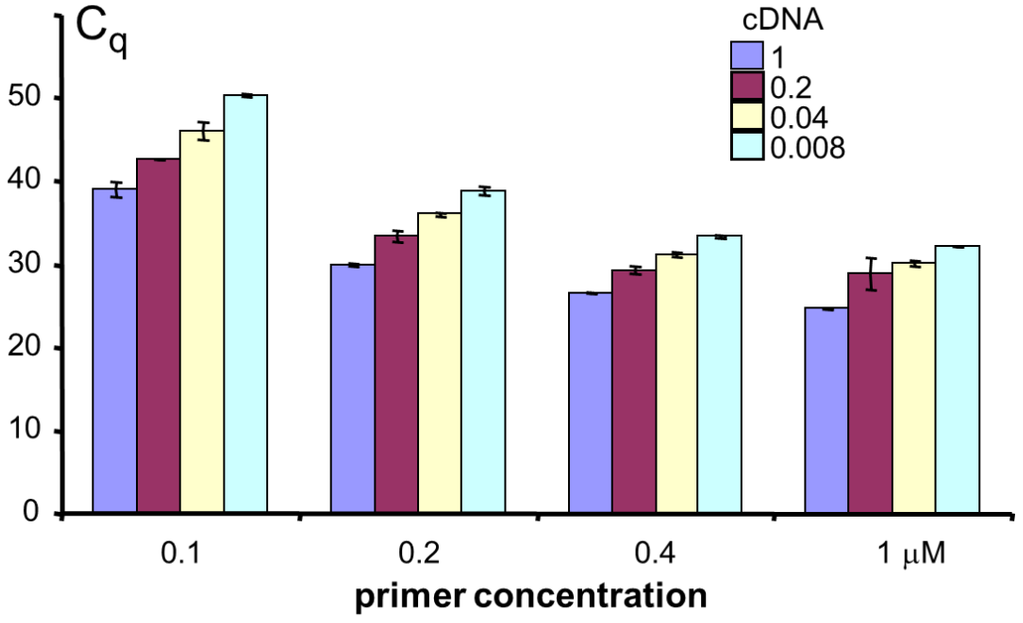


Fig 5B: Significant increase of the observed Cq values with lower cDNA input and higher primer concentration.

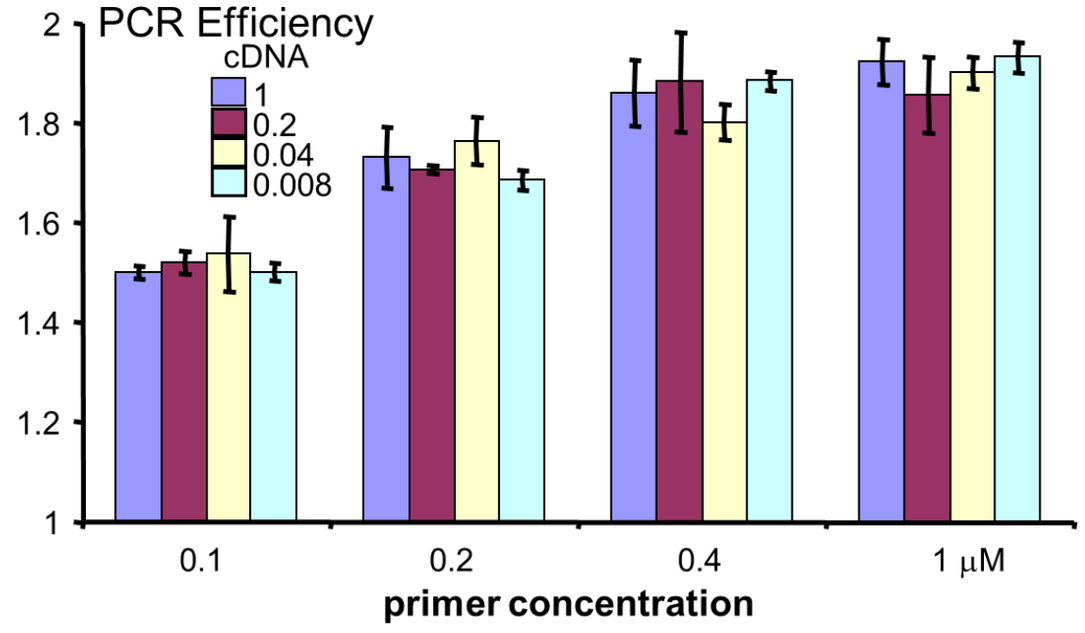


Fig 5C: Significant decrease of PCR efficiency values with low primer concentration but no effect of cDNA input.

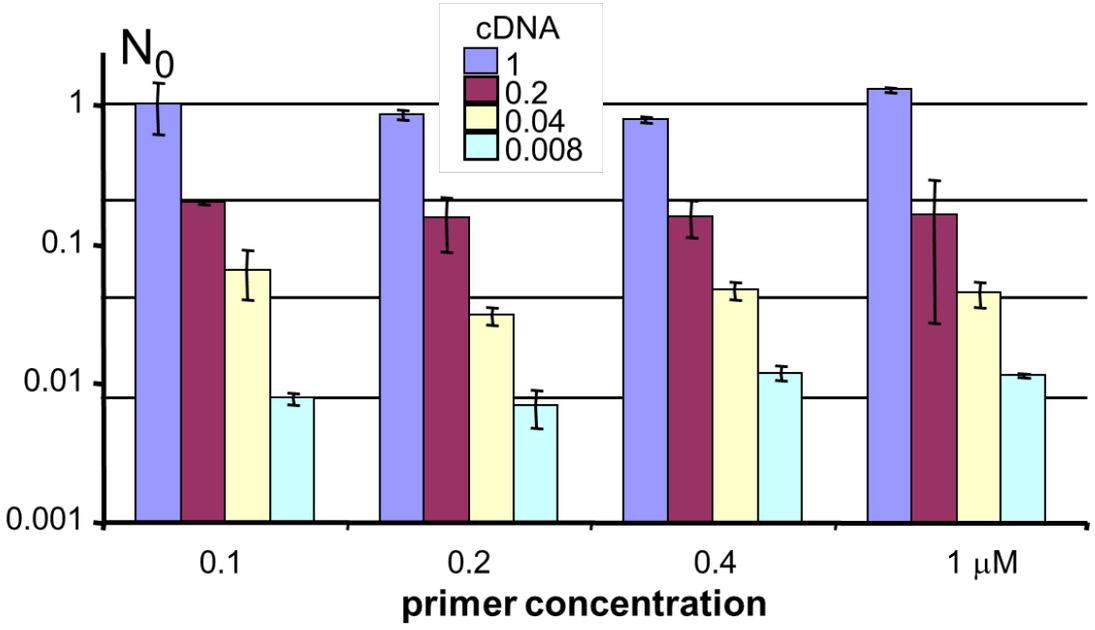


Fig 5D: The resulting starting concentrations (highest input per primer concentration set to 1) calculated with the average PCR efficiency per primer concentration and the Cq value per reaction. The observed target quantities were not significantly different from the values expected in a 5-times dilution series (grey lines).

Section 7

Final recommendations

As suggested by the title of this chapter, an unbiased report of qPCR results should be based on calculations with the assay-specific PCR efficiency (Bustin et al. 2009). Cq or ΔCq values in reports cannot be interpreted objectively without information on the PCR efficiency of each assay. The assumption that all assays are 100% efficient will lead a bias, of which the magnitude and direction depends on the gene expression differences. Derivation of the PCR efficiency from a standard curve requires correct calibration of the pipet used for the dilution series and multiple reactions per dilution to average-out the pipetting error. On the other hand, derivation of the PCR efficiency from the amplification curves avoids the standard curve costs and was shown to lead to similar, but more precise, results (Ruijter et al. 2013). Moreover, the determination of the PCR efficiency from the amplification curve allows a quality check at the level of the individual reactions. The PCR efficiency to be used in the calculations per target is the mean efficiency for all the reactions measuring the respective target in the qPCR experiment. Note, that using the PCR efficiency determined per individual reaction leads to increased variation (Ruijter et al. 2013).

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Chapter 12

Removal of between-plate variation

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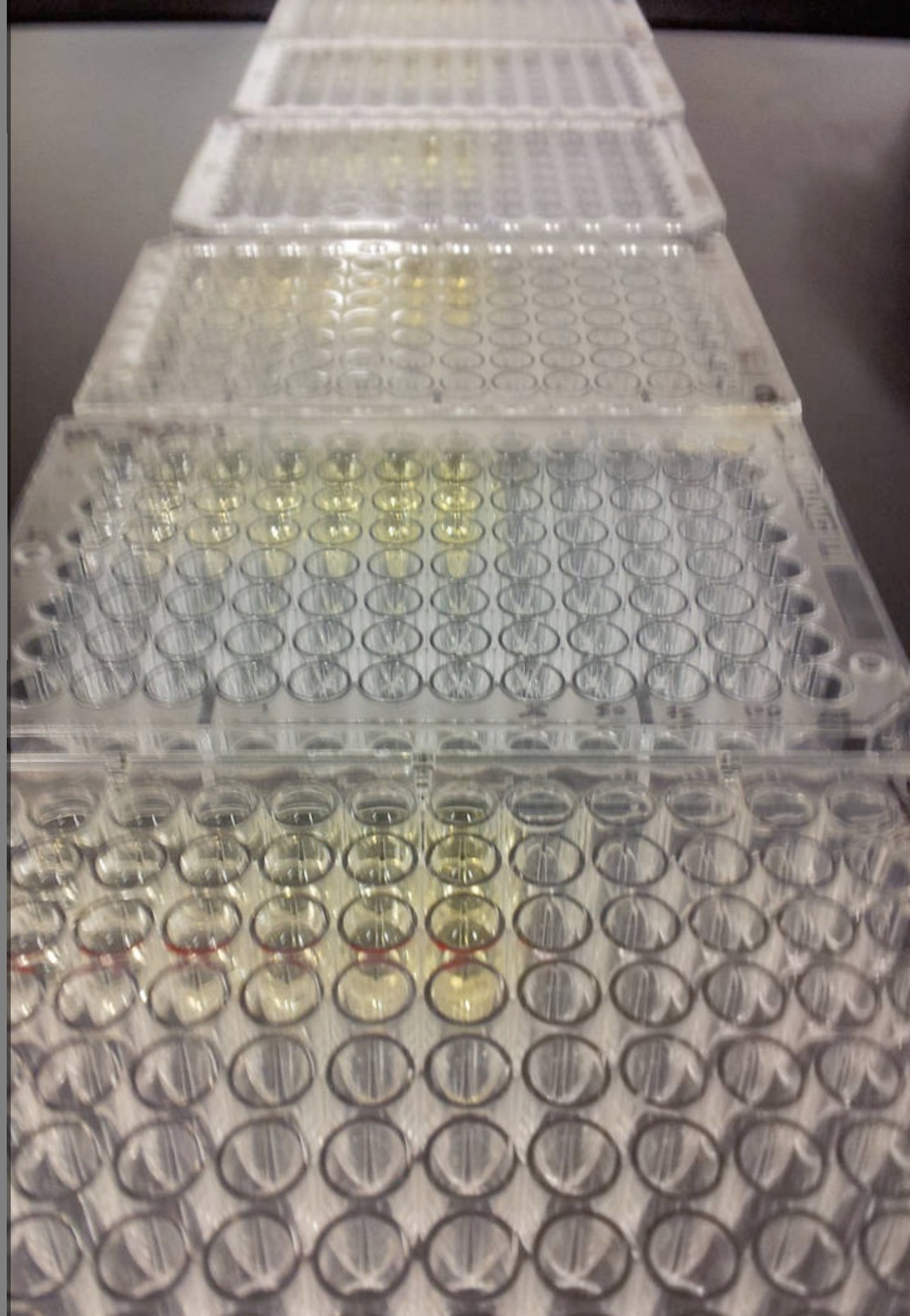

Section 1

Introduction

In experimental biology, replicating a series of measurements under presumably identical circumstances often leads to results that show the same proportional differences between the control and treatment conditions, but different absolute values between different measurement sessions (Hollon and Yoshimura 1989; Ruijter et al. 2006). When multiple quantitative PCR (qPCR) runs are required in a large gene-expression or biomarker discovery analysis, a large variation can also be observed between the different plates in such a multi-plate experiment (Ruijter et al. 2015). This so-called ‘between-plate variation’ may result from unnoticed systematic differences in the yield of the RT-reaction, concentrations of cDNA, primer and/or reagents in the reaction, and the timing of pipetting, denaturing, annealing and elongation phases of the PCR. Such factors affect the observed results per plate systematically: increasing or decreasing all observed target quantities in a plate in the same way. Therefore, the origin of the between-plate variation is generally modelled as a multiplicative factor that systematically biases all values observed on the whole plate. In short, between-plate correction in qPCR data analysis originates from a multiplicative between-plate difference.

Similar multiplicative differences are also observed between the fluorescence measurements on different arrays in a repeated microarray experiment or between sequence counts in different next generation sequencing analyses. In these high-throughput analyses the large number of different target allows correction based

on the assumption that the mean of all measured mRNA or miRNA expression levels, should not differ between the experimental groups (Mestdagh et al. 2009). For each dataset, every observed value is divided by the global geometric mean value in that dataset which sets every dataset to the same scale, and makes them fully comparable. Similar data-driven correction methods, such as quantile, quartile or rank-invariant set correction, can also be used (Bolstad et al. 2003; Tseng et al. 2001; Pradervand et al. 2009). One should keep in mind, however, that these correction methods can only be applied when at least about 50 targets are measured. In qPCR this is most often not the case. Moreover, qPCR targets are chosen because of their possible role in the studied biological or clinical phenomenon and most targets are thus assumed to be regulated and hence to differ between groups.

The between-plate difference in qPCR can be removed by dividing all observed target quantities on a plate by a factor that corrects the inadvertently introduced plate-wide bias. Therefore, the experimental and plate design of the multi-plate qPCR experiment has to allow for the estimation of this correction factor.

Section 2

Statistical model of between-plate variation

Observed target quantities in a multi-plate qPCR experiment can be considered to result from the mixed additive and multiplicative model that was described for multi-session biochemical experiments (Ruijter et al. 2006; Ruijter et al. 2015). The statistical model that describes such multi-session data will be illustrated using a small simulated dataset. This example consists of the observations on 4 different plates of samples resulting from an experiment with 4 genes, each with and without hormone treatment, giving a total of 8 experimental conditions (Supplementary Excel file, sheet 1). Note that an experimental condition is defined as a combination of gene and a treatment. The latter could be an actual physical or chemical treatment but also a difference in biological tissue, clinical disease, developmental stage, etc. Treatment is every variable in the experiment that makes the groups different.

According to the null hypothesis of the statistical test that would be applied to test the effect of a treatment on a gene the effect should be the same in all conditions; the null hypothesis is that there is no effect. The observed value per group is then expected to be equal to the overall mean of all observations (). However, each hormone treatment has its own effect (Ei) and each measurement will suffer from random biological and/or technical error (ε). Each individual measurement (Yi) can thus be modelled as:

$$Y_i = \bar{Y} + E_i + \varepsilon \qquad \text{[Eq. 1]}$$

Because qPCR experiments are aimed at measuring differences in expression patterns between genes, the effect of the gene (Gj) should be part of the additive part of the model:

$$Y_{i,j} = \bar{Y} + E_i + G_j + \varepsilon \qquad \text{[Eq. 2]}$$

Eq. 2 describes the measurements of genes in treated or untreated samples that can be expected when the experiment would consist of only one plate. In a multi-plate experiment, these observations per plate are multiplied by factor per plate (Fk). The full combined multiplicative and additive model can, therefore, be described with Eq. 3:

$$Y_{i,j,k} = F_k\left(\bar{Y} + E_i + G_j + \varepsilon\right) \text{ [Eq. 3]}$$

This model states that any measurement in a multi-plate experiment can be described as result of an additive part: overall mean plus the effect of the treatment plus the effect of the gene plus a random error, multiplied by a factor per plate.

The multiplicative nature of the between-plate variation in the example dataset becomes apparent from the fact that the lines connecting the data points in each run are approximately parallel in a logarithmic plot of the data (Fig. 1A). When the multiplicative factor is not removed from the values in the dataset, the large variation that it adds to the mean gene expression level in the experimental groups will decrease the power of the experiment (Fig. 1B). Therefore, the observed data should be corrected.

Section 3

Correction with inter-plate calibrators

The common approach to remove between-plate variation in large qPCR experiments is the use of calibrator samples (Hellemans et al. 2007; Bustin et al. 2009). To enable the calibrator approach, a number of calibrator samples has to be present on every plate in the experiment. In principle, any sample can be selected as calibrator. Preferably several calibrators should be selected from real biological samples showing high and low expression levels (Hellemans et al. 2007). After the measurement, the geometric mean of the target quantities of the calibrator samples on each plate is calculated and used as correction factor for that plate. To perform the correction, every observed target quantity on a plate is divided by this correction factor. In practice, all observations on a plate are divided by a constant. To illustrate an extreme case, the measurement of gene A, without treatment, was set to be the calibrator measurement in the example dataset. Per plate, all observations were divided by this value (Supplemental Excel file, sheet 1). These calibrator-corrected values are then averaged per condition and plotted (Fig. 1C). Notably, the use of the calibrator leads to loss of the original measurement scale; the mean of the calibrator samples (condition “a” – “no” in Fig. 1) from the different plates becomes 1. Therefore, the calibrator samples can no longer be used in further statistical analysis; their residual error is artificially reduced compared to the variation in the other samples. In the illustrated extreme case, with only one calibrator measurement per plate, the calibrator condition is without variation after the correction (Fig. 1C). This loss of variation of the calibrator illustrates another

negative effect of the calibrator approach. Because no measurement is free of technical or biological error, the measurement error that was present in the calibrator samples has been propagated to the other samples in the plate. Although this is not yet clear when the calibrator approach is compared to the raw data (compare Fig. 1B and 1C) this effect will become clear in the next paragraph. This propagation of error of the calibrator also occurs when more calibrator samples are measured on each plate (Ruijter et al. 2015).

Another drawback of the calibrator approach is that it requires the successful measurement of all calibrator samples. Missing calibrator values on a plate will bias the calibration factor and thus the results reported for that plate; too many missing calibrator values may lead to loss of a whole plate.

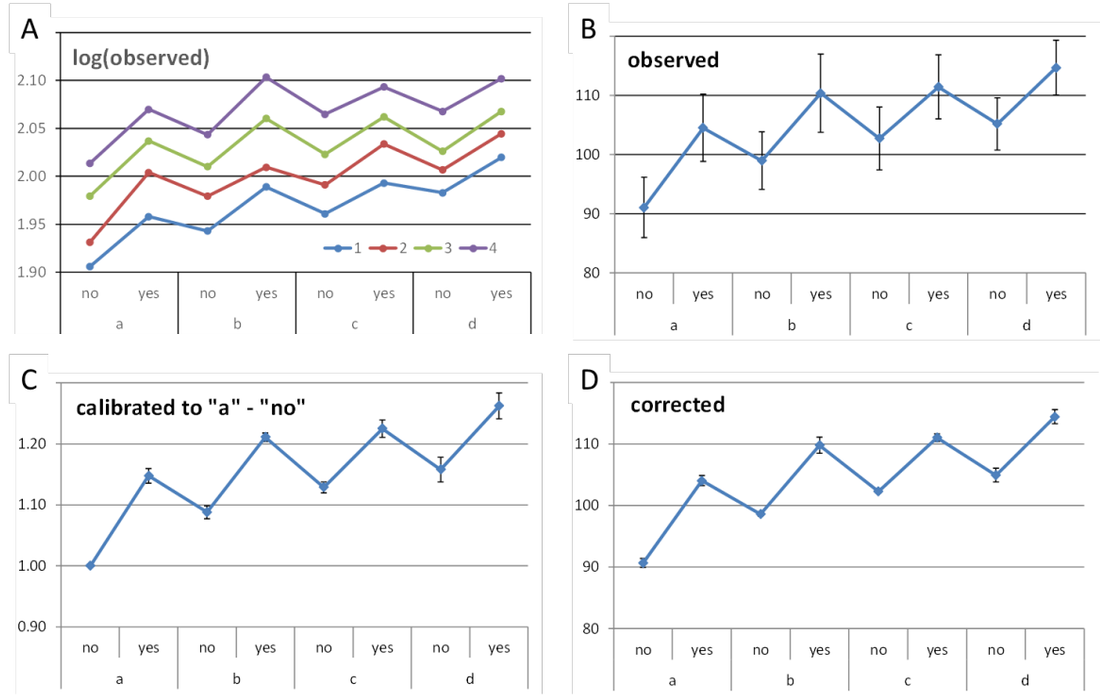

Figure 1: Example dataset to illustrate the removal of between-plate variation. The dataset consists of measurement results of 4 genes (a-d) in tissue samples with or without treatment (yes-no) in four different plates. A. Logarithmic plot of the observed data per plate (coloured lines). The parallel lines indicate the multiplicative difference between plates. B. Mean (and SEM) of the observed data per gene and treatment. C. Mean (and SEM) of the data corrected by using the a-no condition as calibrator. Because on each plate all values are divided by the value of a-no, the mean of this condition becomes 1, without variation. D. Mean (and SEM) of the data after factor-correction. In this correction the original measurement scale and the variation in every condition are preserved.

Section 4

Correction with all overlapping conditions on the plate

To avoid the draw-backs of the use of calibrator samples another correction approach, dubbed factor-correction, was developed (Ruijter et al. 2006; Ruijter et al. 2015). This approach determines the correction factor per plate from all observations on the plate that overlap with observations on one or more other plates in the experiment. Moreover, the factor-correction approach is not based strictly on technical replicates, as is the case for calibrator samples, but also on biological replicates. This means that all target quantities that have technical or biological replicates among the different plates in the experiment can serve to estimate the correction factors per plate. Technical replicates result from replicate measurements of the same biological sample on one or more plates. Biological replicates result from measurement of different biological samples from the same experimental condition. Because these biological replicates are assumed to belong to a normally distributed populations with a common group mean and standard deviation, they will, on average, give the same observed values per group. Therefore, when different values are observed for biological replicates on different plates, this difference also reflects the between-plate multiplicative difference.

As described above, the statistical model of multi-plate observations includes the effects of gene, experimental treatments and random error. For the estimation of the correction factors per plate a further assumption is that the product of the correction factors equals 1. This requirement ensures that the correction factors

(F) can be determined by the so-called ratio approach (Ruijter et al. 2006). This approach starts by calculating the between-plate ratio for a pair of observations from different plates (a and b) but for the same experimental treatment (i) and gene (j):

$$\frac{Plate_a}{Plate_b} = \frac{Y_{i,j,a}}{Y_{i,j,b}} = \frac{F_a(\bar{Y} + E_i + G_j + \epsilon)}{F_b(\bar{Y} + E_i + G_j + \epsilon)} = \frac{F_a}{F_b} \quad \text{[Eq. 4]}$$

In this between-plate ratio, the ratio of the two normally distributed additive parts of the multi-plate model [see Eq. 2], has a Cauchy distribution. For this distribution, describing the ratio of two independent normally distributed random variables, no average or variation can be calculated. However, its symmetrical shape implies that, on average, the 'mean' is equal to 1 and thus the ratio of the additive parts of the multi-plate model cancels out (Ruijter et al. 2006). Therefore, every condition-matched ratio of observations made in 2 plates is an unbiased estimate of the ratio of the correction factors of these plates. Because of this property, the best estimates of the correction factors per plate can be determined by constructing the complete matrix of between-plate ratios for all pairs of observations that have genes and treatments in common. From this matrix the correction factors per plate can be derived (for further details (Ruijter et al. 2015)). Application of this approach to the example dataset shows that the factors per plate range from 0.89 to 1.13 (Supplemental Excel file: sheet 1). After division of the observations per plate by these factors the variation per condition is further reduced (Fig. 1D). The fact that this variation is smaller than the variation after the calibrator approach (Fig. 1C) shows that the random measurement error in the calibrator sample (condition a-no) indeed was propagated over the other conditions.

Plate design required for between-plate correction

In qPCR experiments, the variables that the researcher wants to compare are the targets, experimental treatments and clinical or biological tissues. A plate design that allows an estimation of the correction factors should, therefore, consists of plates that at least overlap with respect to some of those variables. The more overlap, the more efficient the correction factors can be estimated.

In the calibrator approach, a limited number of calibrator samples is selected, or added, to enable calculation of a correction factor per plate. The overlap between plates consists of the replicate measurements of the calibrator samples on the different plates. The correction factor per plate is calculated as the geometric mean of the observations on the calibrator samples. Preferably, calibrator samples should be chosen from different experimental groups and from targets with a range of expression levels similar to those of the studied samples.

Plate design in qPCR experiments often aims at sample maximisation: biological or medical samples that have to be compared are measured all in 1 plate, limiting the number of targets that can be measured on that plate (Hellemans et al. 2007). Such an approach allows comparison between samples or sample groups, but not between targets. An example from an experiment with two large groups of patients and controls, in which four genes of interest are measured in triplicate serves to illustrates this plate design. Although the total number of required reactions would fit on six 384-wells plates (Fig. 2) the calibrator samples that are needed means that the sample maximisation design would require more

plates to ensure that every gene can be compared between all individuals in the two groups (Fig. 2A).

Alternatively, target maximisation (all targets but a limited number of tissue samples on each plate) enables comparison between genes but hampers comparison between samples. The calibrator samples that are needed in this design make that already more than three plates are required to enable combination of all measurements per group (Fig. 2B). Extra calibrators would be required to enable the comparison of the two groups.

In the factor correction approach, the required overlap is not restricted to such technical replicates. The statistical model on which the correction is based requires maximum overlap between plates with respect to conditions, consisting of the target genes and samples collected from the same clinical or biological tissues under the same experimental treatments. Overlap between plates is, therefore, based on biological as well as technical replicates. The maximum overlap design that forms the basis of factor-correction allows comparison of samples and targets and thus enables the study of gene expression pathways with a multi-plate experiment. All measurements of the example dataset fit on six plates (Fig. 2C). By alternating the groups of samples systematically over the four different genes, each plate partly overlaps with 2 of the other plates.

The experimental design of a multi-plate experiment should preferably be balanced and complete: a similar number of samples coming from every condition on each of the different plates. Because every condition then has the same influence on the between-plate correction, the loss of condition effects through the correction is avoided. Such a loss of effect would occur when different conditions are measured on different plates: the condition effect can then not be distinguished from the plate effect. However, factor correction can be applied to incomplete designs.

The advantage of using all overlap between plates is that measurements that have failed for technical reasons do hardly affect the factor estimation. Moreover, these lost measurements can be redone easily in a new run and the new plate can then be added to the experiment. Similarly, experimental conditions or targets-of-interest that turn out to be required to complete the biological experiment can be added. A new plate, partly overlapping with (some of) the earlier plates enables the estimation of new correction factors that allow the combination of all plates in the complete experiment. This makes the factor-correction approach very flexible in the long run: it can still be used to extend an experiment even when the store of calibrator samples has run out.

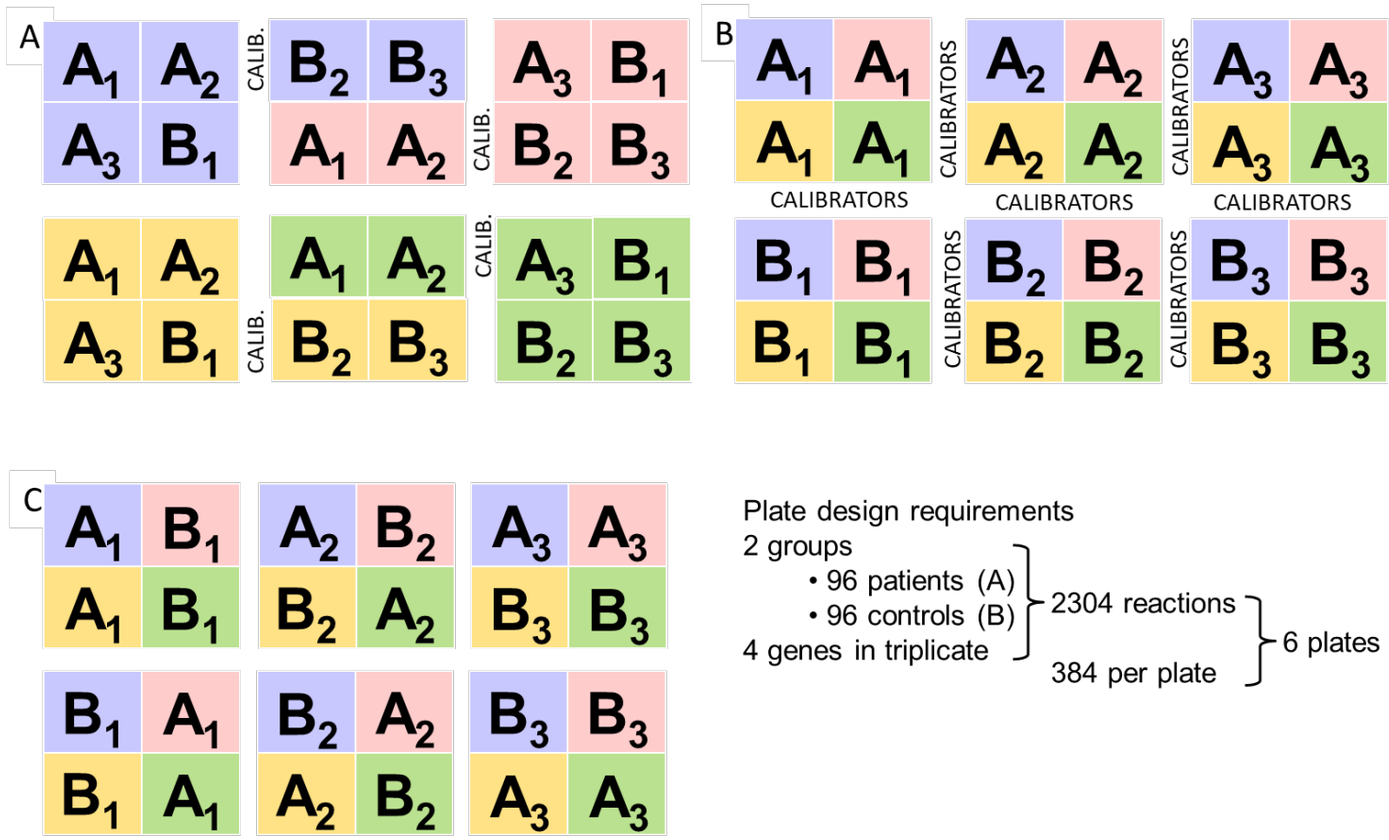

Figure 2: Plate design for a large-scale qPCR experiment. The experiment consists of measurement of four genes (coloured rectangles) in two groups (A and B). The samples are measured in triplicate. A. Sample maximisation. This design aims at comparison between samples per gene. Because there are too many reactions to fit on one plate calibrators are required to allow comparison of all samples of the two groups. B. Target maximisation. This design aims at the comparison between targets. Calibrators are required to comparison of targets within each group. C. Overlap maximisation. Without extra calibrators the between-plate correction can be performed and the sample groups and targets can be compared.

Section 6

Example of the use of the correction program: Factor-qPCR

Calculating the correction factors by hand can be done for small examples like the one shown in Fig. 1, but becomes very cumbersome when the number of treatments, targets and plates increases. The factor-correction program, was made available for general use in multi-session experiments (Ruijter et al. 2006). A version of this program specifically for qPCR experiments, Factor-qPCR, was presented at the qPCR & NGS 2015 Event and released almost 10 years later (Ruijter et al. 2015). The Supplemental Excel file shows an example of the use of this program. Short instructions on the use of Factor-qPCR are given in sheet "sheet_2_use_of_Factor_qPCR". The Factor-qPCR program contains a more extensive 'Help function' and details on its use are given in the paper (Ruijter et al. 2015).

The example dataset consists of the observations from six qPCR plates that were analysed with LinRegPCR (Ruijter et al. 2009). The Excel file contains the analysis results of this program for only the four genes that were considered as reference genes (sheets Run 1 to Run 7, Run 4 failed). When these data are read into Factor-qPCR, the correction between plates can be performed and the corrected target quantities can be exported to Excel (sheet: "Results of Factor-qPCR"). The factors per plate range from 0.53 till 2.63 representing a more than five-fold difference between plates. The graphs made from the observed and corrected target quantities show that the coefficient of variation in the expression levels per gene and tissue was halved (Fig. 3A and 3B). When the corrected data per plate are

plotted as separate line this example shows that after correction by a constant factor per plate, all between-plate differences that are not multiplicative will still be present (Supplemental Excel file sheet: “Between_plate_graphs”).

When the researcher adds an annotation column to label the calibrator samples to the input of Factor-qPCR, this program can also be used to perform the calculations for the calibrator approach. To demonstrate this, a column was added to the LinRegPCR results, defining the PE tissue as the calibrator tissue (Supplemental Excel file, sheets Run 1 – 7). This column has to be added to the data range read into Factor-qPCR and can then be used to ensure that the correction factors are based on the calibrator samples only (see Instructions on sheet 2). The results of this calibrator correction show that the variation in the calibrator samples themselves is reduced but that this is not the case for the overall CV in the dataset s a whole: the overall CV has hardly declined (Fig. 3C). This shows that the variation in the calibrator samples has been distributed over the other tissues. The latter effect is clearly visible when the corrected data are plotted per plate (Supplemental Excel file sheet: “Between_plate_graphs”).

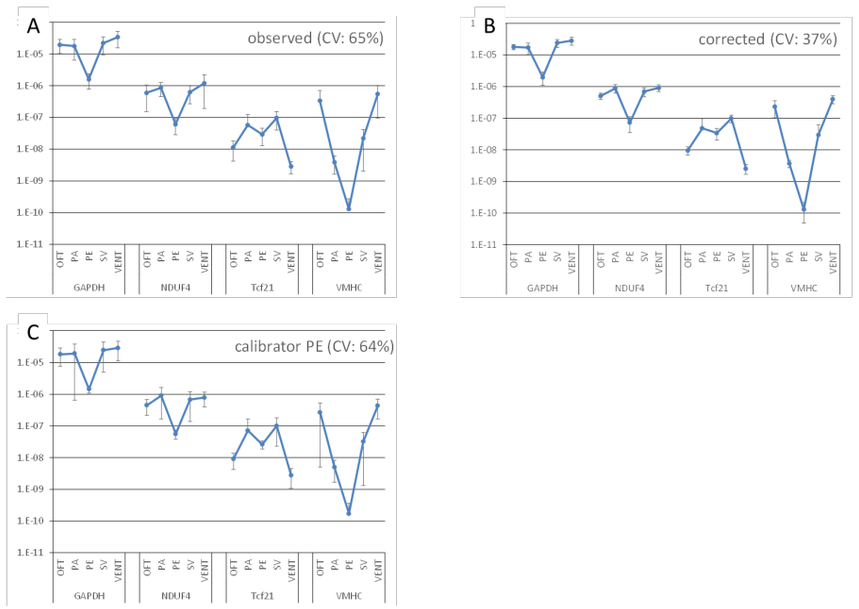

Figure 3: Example of between-plate correction with Factor-qPCR. The graphs show the mean target quantities (and SD) of the reference genes of in five tissues. A. Observed data. B. Factor-corrected data. C. Data after correction with the PE sample used as calibrator, preserving the difference between targets. The coefficient of variation (CV = SD/mean) was calculated per condition and the geometric mean per panel is reported.

Final recommendations

Because between-plate variation is multiplicative, the removal of this variation can be done by dividing all observed values on a plate by a correction factor calculated based on observations on that plate. One approach is to include the same set of samples on every plate and to use these samples as calibrators: the geometric mean of their observed values is the correction factor. Because this approach is sensitive to missing values in the calibrators and the correct measurement of the calibrators, we introduced Factor-qPCR. In this method there is no need for additional calibrator samples because all measurements from conditions that overlap between plates are used to determine the correction factor per plate. To optimally apply this method to a multi-plate experiment, a plate design called “overlap maximisation” is preferred. To easily obtain this overlap, it is recommended to systematically distribute technical replicates over different plates.

Section 8

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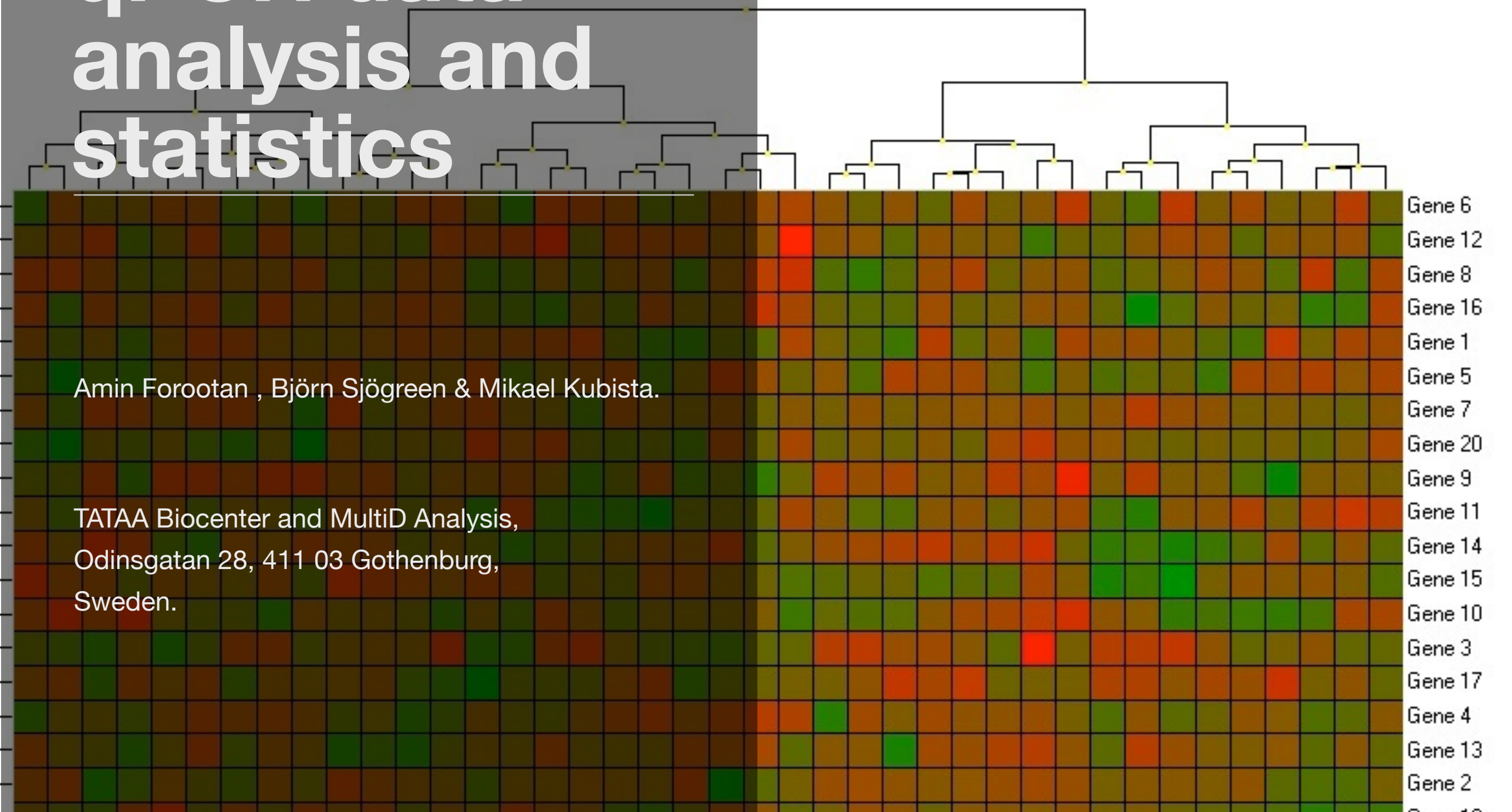
Chapter 13

MIQE compliant qPCR data analysis and statistics



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Summary

qPCR analysis has emerged as the golden standard analysis method for the quantification of nucleic acids. The technique is robust, sensitive and specific making quantification more reliable and accurate than with alternative technologies (Kubista et al., 2006). Proper quantification requires that all sources to uncertainty in the measurements are considered when estimating concentrations and are indicated in the reported values as confidence intervals. In this chapter we teach proper MIQE compliant (Bustin et al., 2009) statistical analysis of qPCR data from the import of measured data from the qPCR instruments, data pre-processing and data analysis. All three methods of quantification: absolute quantification, relative quantification and expression profiling are covered.

Section 1

Data arrangement

When groups are compared, data are classically arranged with the measured Cq (explained variables) in columns headed with the experimental group label (explanatory variables). This arrangement provides easy overview of the data (Figure 7.1). However, it is not practical for advanced studies that may include more than one nominal factor or covariate (variable of metric character, e.g. time, age, dose etc.), multiple markers, replicate measurement, repeated measurements (same subject sampled repeatedly), multiplate measurements, etc. A more flexible approach is to arrange the data with samples as rows and all variables in columns (Figure 7.1). The format is readily generalized to any number of markers and additional columns and rows can be added that specify the experimental design, indexing samples, markers, plates etc. These are referred to as classification columns and classification rows and have labels starting with #. In the example shown in Figure 7.2, #Repeat indexes qPCR technical replicates (samples with the same index are replicates on the qPCR level). These are expected to be highly similar and shall be averaged during data pre-processing.

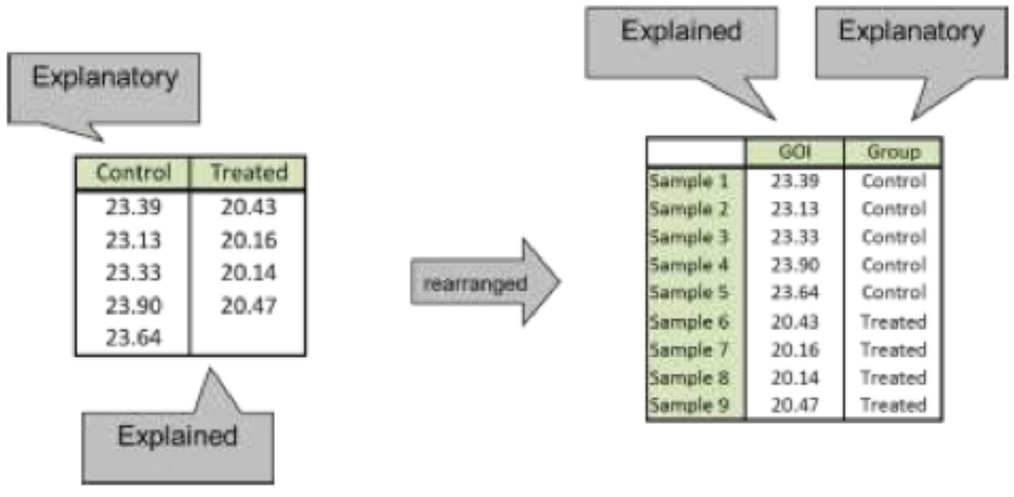


Figure 7.1 - Traditional arrangement of data with experimental group labels in column's heads (left) and modern, generic arrangement of data with one variable per column (right).

	MG	RG	#Repeat	#Treatment	#Pairing
S1	22.22481...	16.71773...	1	1	1
S2	17.93896...	16.59702...	1	1	2
S3	19.76137...	16.60889...	2	1	3
S4	17.23068...	16.23785...	2	1	4
S5	16.57810...	16.89222...	3	1	5
S6	18.25507...	16.54029...	3	1	6
S7	29.47699...	16.86087...	1	2	1
S8	18.68488...	16.08867...	1	2	2
S9	22.91167...	16.67638...	2	2	3
S10	17.80890...	16.15741...	2	2	4
S11	16.80731...	16.05589...	3	2	5
S12	19.77758...	16.19985...	3	2	6
#Gene	0	1			

Figure 7.2 - Example of data arrangement in GenEx. First column list the samples. 2nd and 3rd columns are measured Cq values. 4th column indexes technical replicates, 5th column indexes treatment groups and 6th column indexes paired samples. Bottom row identifies maker and reference genes.

#Treatment indexes treatment groups that eventually shall be compared using a statistical test. Finally, the study is paired, meaning that each subject received both treatments and a sample was collected after each treatment. Paired study designs are more powerful, because the pairing reduces confounding variation. This elevates the power of the test and the experiment requires fewer subjects. It can be, for example, samples collected from all subjects before treatment and a second set collected after treatment. It can also be positive and negative samples collected from the same subject or genetically similar individuals such as siblings, identical twins or clones. A special type of paired study design is repeated samplings, used in more than two subsequent measurements. In general, the word "paired" is replaced by "repeated". Specialized statistical procedures are available to analyze repeated samplings.

Section 2

Data import

The experimental design is defined in part by deciding on the experimental factors and covariates involved in the experiment and in part when the samples and assays are mixed while dispensed into the qPCR containers. This information is critical for proper analysis and mining of the measured data. This has been realized by several of the leading qPCR instrument and assay providers. Roche LC480 software for RealTime Ready custom and focus panels, for example, names all genes and samples, indicates reference genes, and specifies technical and biological replicates at various levels. This information is transferred to GenEx and arranged appropriately for downstream analysis using a wizard. A similar high level user friendly solution is provided by Exiqon, who offers a customized version of GenEx with a powerful wizard to read their miRCURY LNATM Universal RT microRNA PCR platform. On the BIOMARK microfluidic platform from Fluidigm, technical and biological replicates are indicated by the naming of assays and samples, and appropriate classification columns are created automatically. Data generated on other qPCR instruments can also be read by GenEx, including the Stratagene MX300X from Agilent, Realplex from Eppendorf, CFX96/384 from Bio-Rad, Eco from Illumina, and the many different qPCR platforms from Life Technologies. These efforts from the instrument manufacturers to transfer experimental design information automatically or at least semi- automatically into GenEx substantially simplify the pre-processing needed to prepare qPCR data for statistical analysis.

Section 3

Data pre-processing

INTRODUCTION

1. Interplate Calibration
2. Efficiency corrections
3. Normalize using spiking
4. Normalize to sample amount
5. Average qPCR replicates
6. Correct for genomic DNA background
7. Normalization with reference genes
8. Average technical replicates
9. Normalize with reference sample(s)
10. Relative quantities
11. Convert to log scale

For most studies performed today the ΔΔCq method is not sufficient to analyze qPCR data. Not that we are calculating differently, but the studies have become larger and the experiments more complex. In fact, for most studies performed today, it is not even possible to write a closed form expression to calculate the resulting expression response. Rather the measured data must be processed sequentially to account for the various aspects of the experiment. In particular, it is essential to correctly define the statistical unit (often referred to as a subject when organisms are used). Each unit should be associated with a single value for each variable to use common statistical methods. This value must, however, frequently be assembled from various measurements, i.e. responses of target and reference genes, estimated amplification efficiencies, etc. In order to integrate the process into a logical workflow, GenEx provides an intuitive wizard with the following sequential operations:

Interplate calibration

Many studies cannot be fitted in a single experimental run or for practical reasons have to be extended over time. qPCR instruments perform base-line correction and set threshold separately for each run, which introduces a bias between the Cqs measured in different runs. This bias can be compensated for by performing a common amplification in all plates, where the same sample is analyzed for a given assay. This sample is called Inter-Plate Calibrator, IPC. Any variation in the measured Cqs of the IPC among runs reflects systematic variation due to

instrument factors and should be compensated for. It is sufficient to run a single IPC for each channel in the instrument if a common threshold and base-line correction is used. It is not recommended to perform separate inter-plate calibrations for each target. Since every correction adds confounding variation to the data, unimportant corrections shall be avoided, as they may impair data quality rather than improving it. For the same reason, it is a good strategy to use a robust sample for IPC and analyze it in replicates. In multiplate experiments, the runs and the inter-plate calibrators shall be indexed in classification columns.

Efficiency correction

If PCR efficiency has been estimated, the measured Cq values can be corrected to account for suboptimal amplification. Typically, PCR efficiencies are estimated from serial dilutions run separately. The PCR efficiencies may then be listed in a classification row for automatic correction in GenEx.

Normalize using spiking

PCR efficiency depends on the sample matrix. Usually it is assumed that the sample matrix and thus the PCR efficiency is constant. But occasionally there are variations, which can be tested for using an exogenous spike added to the samples. Differential expression of the spike between the test and a standard sample reflects the sample's specific inhibition and can to some degree be accounted for.

Normalize to sample amount

Measured Cq values depend on the sample input. This can be the sample volume processed, amount of RNA used for reverse transcription, or cell count. If sample inputs vary, data may have to be normalized. The sample input shall be indicated in a classification column.

Average qPCR replicates

If qPCR replicates are available they shall be indexed in a classification column and their Cq values shall be averaged.

Correct for genomic DNA background

When quantifying RNA levels using RT- qPCR, the assays may also amplify genomic copies of the target if the DNase treatment used is insufficient. The amount of genomic background can be assessed by measuring either NoRT controls or by using the ValidPrime approach. The contribution to Cq from the genomic background can be calculated and the Cqs corrected.

Normalization with reference genes

In expression studies normalization to endogenous controls, such as stably expressed reference genes, is popular. In GenEx, you can normalize to any number of reference genes; you can even normalize sets of reporter genes to sets of reference genes to match the genes' properties such as expression levels, stabilities, distribution in tissues, etc. It is also possible to normalize to the mean expression of all the genes (global normalization). Optionally, reference genes can be indexed in a classification row

for automatic processing. Normalization to the expression of reference genes corresponds to calculating ΔCq in the classical approach.

Average technical replicates

If additional technical replicates are available, such as RT, extraction, and sampling replicates, they shall be indexed in classification columns and averaged.

Normalize with Reference Sample(s)

In some paired designs, systematic variation can be reduced by normalizing to the paired sample during pre-processing.

Relative quantities

An arbitrary reference level is selected (which corresponds to ΔΔ Cq in the classical approach) and data are converted to linear scale ($2^{-\Delta\Delta}$ Cq in the classical approach). The reference level can be the most expressed sample, the least expressed sample, mean expression of all the samples, mean expression of a group of samples, or percentage (sum of the expression in all samples set to 1). It is also possible to convert the ΔCq values to an arbitrary linear scale ($2^{-\Delta}$ Cq).

Convert to log scale

For statistical analysis with parametric methods, the data shall be converted to logarithmic scale. Available options are log2, log10, ln, and log(X+1).

All the steps in the workflow are not needed, since some cancel the effect of others. The appropriate steps depend on the experimental design, the controls and references that are available, and the analysis that will be performed.

In addition to the pre-processing work flow, GenEx has correction for missing data. GenEx recognizes two types of missing data, random missing (failed experiments) and non-random missing (off-scale data). There is built in handling of random missing data among technical replicates, which are replaced based on available information, in the course of the pre- processing. This is very useful, since the missing information can be ignored and is automatically accounted for. There are also several means to handle non-random missing or off scale data that are due to too low target amounts, which may bias the biological effect and invalidate the statistical inference in the majority of the statistical tests employed. Outliers in the data can be tested for based on standard deviation and the Grubb's outlier test.

The pre-processing of data is logged and stored in a log file.

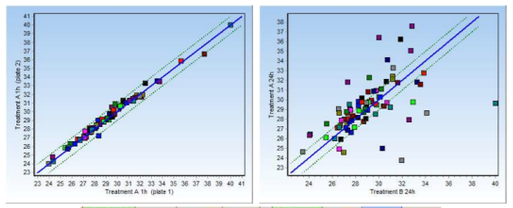
Section 4

Screening by correlation

Several companies, including Roche, Exiqon, Life Technologies, Lonza, Qiagen and TATAA Biocenter, offer pre-plated assays for smooth expression profiling and screening purposes. Data from those plates are readily read into GenEx. Rarely are all assays relevant for every study and a strategy is to analyze a few representative samples of each kind in a pilot study to identify differentially expressed genes to be used in a larger downstream study. This is readily done using the GenEx scatter plot (Figure 7.3). Replicated measurements can be compared to test the reproducibility between plates (top left), or screen for differentially expressed genes under two conditions (top right, bottom). Correlations between genes' expressions can be quantified by calculating the Pearson or Spearman correlation coefficients. This is typically applied to larger number of samples and has, for example, been used to reveal correlations between genes expressed in individual cells (Stahlberg et al., 2010).

Preparing the data for analysis

Groups for comparison are created using the GenEx Data Manager. Treatment groups or treatment factors in multifactorial studies, such as studies of the effect of gender or covariates such as age, time, or drug load, can be indexed in classification columns and used to assign subjects into groups automatically. The groups are assigned colors and symbols for plotting. A neat feature is that colors and symbols can be set independently, which makes it possible to assign subjects to multiple groups and identify these in plots by the shape, size, and color of the symbol. Even shades of colors can be used creatively to indicate various levels of covariates at ordinary scale (e.g. darker shade indicates higher drug load).



Over-exp...	Treatm...	Treatme...	Diff
gene 95	36.4	30	6.41
gene 37	37.61	32.84	4.77
gene 83	31.81	27.34	4.47
gene 42	36.27	31.87	4.39
gene 61	34.13	30.75	3.38
gene 32	32.32	29.17	3.15
gene 29	29.07	26.54	2.52
gene 9	26.46	24.13	2.34
gene 65	35.05	32.73	2.31
gene 49	26.33	24.05	2.28
gene 71	33.3	31.21	2.08
gene 96	29.55	27.48	2.06
gene 39	28.64	26.61	2.04
gene 16	27.55	25.54	2.01
gene 94	30.86	29.09	1.76
gene 73	30.2	28.54	1.67
gene 2	29.84	28.27	1.56
gene 59	28.75	27.21	1.53
gene 5	29.05	27.52	1.53
gene 54	28.95	27.57	1.38
gene 17	28.66	27.31	1.35
gene 18	30.9	29.57	1.33
gene 53	32.41	31.16	1.26

Under-ex...	Treat...	Treat...	Diff
gene 24	29.64	40	-10.36
gene 55	23.73	31.93	-8.21
gene 12	28.66	34.09	-5.43
gene 78	24.97	30.32	-5.36
gene 8	27.92	32.58	-4.66
gene 93	29.78	32.94	-3.15
gene 85	24.59	27	-2.4
gene 81	31.61	33.59	-1.98
gene 84	29.25	30.96	-1.72
gene 70	24.93	26.61	-1.68
gene 34	31.82	33.3	-1.48
gene 51	28.54	29.7	-1.17
gene 63	29.36	30.47	-1.11
gene 44	29.32	30.41	-1.08
gene 60	32.8	33.87	-1.07

Figure 7.3 - Scatter plot comparing replicate measurements performed in separate plates (left) and two different conditions (right). Differentially expressed genes are tabulated (bottom)

In the data manager, subjects and genes can be removed temporarily from analysis to compare results based on analyses of subsets of data. Data can also be mean centered (subtraction of the mean value) or autoscaled (subtraction of mean followed by division with the standard deviation) to change the weights of the genes/samples in analyses. This is particularly useful in expression profiling analysis, where genes having different expression levels can be assigned equal weights. For analyses that apply models based on measured data, such as the standard curve, reverse calibration, neural networks, self organized map, potential curves etc., samples (and genes) can be assigned either training or test. Training data are used to create the model, which is applied to classify the test samples. The various analyses available in GenEx are listed in Table 7.1.

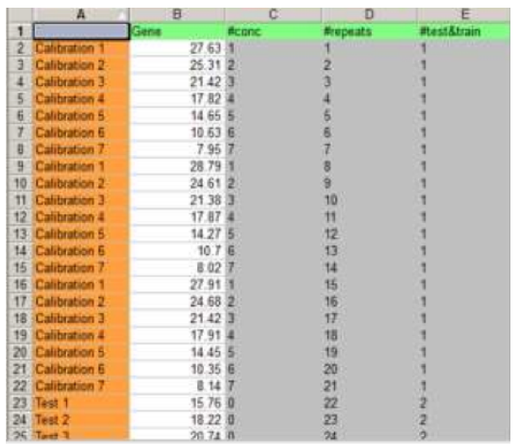
		GenEx Std	GenEx Pro	GenEx Ent.	
Pre-processing of Data					
	Interplate Calibration	X	X	X	
	PCR Efficiency Correction	X	X	X	
	Normalize to Sample amount	X	X	X	
	Normalize to Reference Genes/Samples	X	X	X	
	Normalize to Technical Replicates(e.g qPCR, RT)	X	X	X	
	Normalize to Spike	X	X	X	
	Missing data Handling & Primer-dimer Correctic	X	X	X	
	ValidPrime	X	X	X	
	RT-Correction	X	X	X	
	Test for Outliers	X	X	X	
	Negative Control	X	X	X	
	Internal amplification control	X	X	X	
	Expression profiles (Line plot)	X	X	X	
	Gene quality (Bar plot)	X	X	X	
	Hemolysis plot		X	X	(Only available with Exiqon Wizard)
	Spread of genes (Boxplot)	X	X	X	
	Relative Quantities & Fold Changes	X	X	X	
Finding Optimal Reference Genes		GenEx Std	GenEx Pro	GenEx Ent.	
	geNorm	X	X	X	
	NormFinder	X	X	X	
Plots		GenEx Std	GenEx Pro	GenEx Ent.	
	Scatter plots	X	X	X	
	Line plots	X	X	X	
	Bar Plot	X	X	X	
	Box and Whiskers Plot	X	X	X	
Principal Component Analyses		GenEx Std	GenEx Pro	GenEx Ent.	
	PCA		X	X	
	P-Curve			X	
	Dynamic PCA			X	
Cluster Analyses		GenEx Std	GenEx Pro	GenEx Ent.	
	Hierarchical Clustering/Dendrogram	X	X	X	
	Heatmap Analysis		X	X	
Networks		GenEx Std	GenEx Pro	GenEx Ent.	
	Selforganizing MAP (SOM)		X	X	
	Artificial Neural Network (ANN)			X	
	Support Vector Machine (SVM)			X	
Regression Analysis		GenEx Std	GenEx Pro	GenEx Ent.	
	Standrad Curve	X	X	X	
	CLSI EP6- Linearity test			X	
	Reverse Calibration		X	X	
	Limit of Detection		X	X	
	Partial List Square			X	
Statistics		GenEx Std	GenEx Pro	GenEx Ent.	
	Descriptive Statistics	X	X	X	
	Parametric t-tests	X	X	X	
	Non-parametric tests	X	X	X	
	One-way ANOVA	X	X	X	
	Two-way ANOVA		X	X	
	Nested ANOVA		X	X	
	Histogram		X	X	
	Boxplot Groups	X	X	X	
Correlation		GenEx Std	GenEx Pro	GenEx Ent.	
	Spearsman Rank Correlation Coefficient	X	X	X	
	Pearson Correlation Coefficient	X	X	X	
	Scatter plot (samples, Genes, Groups)	X	X	X	
Experimenta Design		GenEx Std	GenEx Pro	GenEx Ent.	
	Sample Size		X	X	
	Experimental Design Optimization			X	
Diamostics		GenEx Std	GenEx Pro	GenEx Ent.	
	Survival Analyse			X	
Quality Ctrl		GenEx Std	GenEx Pro	GenEx Ent.	
	Uniformity test			X	
3-Way		GenEx Std	GenEx Pro	GenEx Ent.	
	3-Way analyse			X	
Wizards		GenEx Std	GenEx Pro	GenEx Ent.	
	Import Wizard	X	X	X	
	Exiqon Wizard		X	X	Require additional license
	LT Wizard		X	X	Require additional license
	Roche Wizard		X	X	Require additional license
	Olink Wizard		X	X	Require additional license

Table 7.1 - Analyses available in different versions of GenEx.

Section 6

Standard curve and reverse calibration

Amounts of pathogens in field samples can be quantified using qPCR by comparing the measured Cqs of the field samples with those of standard samples by means of a standard curve. Representative data arrangement is shown in Figure 7.4. In addition to the measured data, the concentration of the standards is given in a classification column. Additional classification columns can be used to index replicates and to identify the standard and test samples. Any technical replicates shall be averaged during pre-processing and the averaged Cq shall be considered a single data point. Independently prepared standards are treated as different data points, while replicate measurements of field samples are averaged and used as a single more precise estimate. Groups are created in the GenEx Data Manager and assigned either test or training status. Samples can also be reversibly removed from analysis (Figure 7.5). A confidence level is set for the analysis.



	A	B	C	D	E
1		Gene	#conc	#repeats	#test&train
2	Calibration 1	27.63	1	1	1
3	Calibration 2	25.31	2	2	1
4	Calibration 3	21.42	3	3	1
5	Calibration 4	17.82	4	4	1
6	Calibration 5	14.65	5	5	1
7	Calibration 6	10.63	6	6	1
8	Calibration 7	7.95	7	7	1
9	Calibration 1	28.79	1	8	1
10	Calibration 2	24.61	2	9	1
11	Calibration 3	21.38	3	10	1
12	Calibration 4	17.87	4	11	1
13	Calibration 5	14.27	5	12	1
14	Calibration 6	10.7	6	13	1
15	Calibration 7	8.02	7	14	1
16	Calibration 1	27.91	1	15	1
17	Calibration 2	24.68	2	16	1
18	Calibration 3	21.42	3	17	1
19	Calibration 4	17.91	4	18	1
20	Calibration 5	14.45	5	19	1
21	Calibration 6	10.35	6	20	1
22	Calibration 7	8.14	7	21	1
23	Test 1	15.76	0	22	2
24	Test 2	18.22	0	23	2
25	Test 3	20.74	0	24	2

Figure 7.4 - Data prepared for analysis using a standard curve. Classification columns indicate concentrations of standards, sample replicates, and indexes test and training samples.

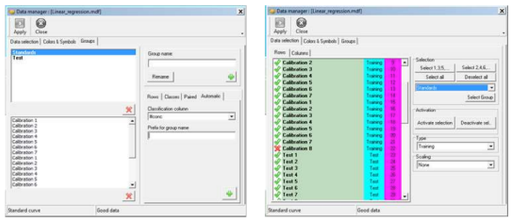
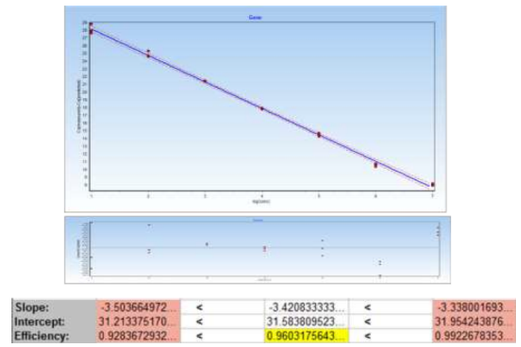


Figure 7.5 - Groups are created in the GenEx data manager optionally using information in classification columns (left). Samples are assigned training or test status, and may be reversibly inactivated (right).

The standard curve is the best straight line fit of the Cqs measured for the standard samples to their concentration in logarithmic scale (Figure 6). It is calculated using linear regression and defines the intercept, which is the Cq expected for a sample containing a single template molecule, and the slope. From the slope, the PCR efficiency is estimated. GenEx also calculates the uncertainties in the estimates of the slope and the intercept, which are reflected by the dashed lines in the plot as the Working-Hotelling area, and the confidence interval for the PCR efficiency (Figure 7.6). It is essential to calculate the confidence information since it reflects the precision of the estimated efficiency. In this example, the precision of the estimated efficiency is quite high, because a large number (21) of standards was used and a wide concentration range was covered (6 logs). In the literature, we frequently see standard curves based on a substantially lower number of standards. The PCR efficiencies estimated from such standard curves are highly uncertain and any corrections made are unreliable.

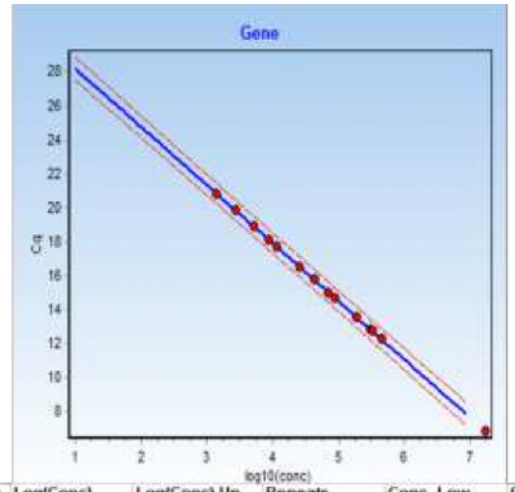
Figure 7.6 - Top: qPCR standard curve (blue line) with uncertainty indicated (dashed red lines). Center: Residual plot showing the deviations of the standard points from the fitted standard curve. Bottom: estimated slope, intercept and PCR efficiency for the standard curve including confidence intervals.



The residual plot shows the deviations of the standard samples' measured Cq and their predicted Cq by the standard curve (Figure 7.6). If the straight line standard curve is adequate to model the data, residuals should fluctuate randomly. If the model is inadequate, runs of positive and negative residuals will be seen. GenEx performs a statistical test for the number of runs, and if they are too few, warns that the linear standard curve may be an inadequate model. Outliers are readily identified visually in the residual plot and GenEx further uses the Grubb's test to support

the removal of outliers. In general, no more than one outlier should be removed from a standard curve (EP6-A, Vol 23, no 16 (2003). Approved guideline NCCLS). If multiple outliers are indicated, the approach used is likely to be unstable and should be overseen. When replicates are available, the residual plot also reveals if noise increases at low concentrations.

A reliable standard curve is critical for accurate estimation of the concentrations of field samples, which in GenEx are referred to as test samples. The estimates improve if the field samples are available in replicates that can be averaged to reduce confounding variation. Concentrations of the unknown samples are estimated by entering the standard curve at the measured Cq and reading out the log of the concentration on the x-axis (Figure 7.7). The Working-Hotelling area, which reflects the prediction uncertainty, is wider than before because of the additional error contribution from the measured Cq. GenEx calculates confidence intervals for the estimated concentrations. The confidence intervals are symmetric around the mean in logarithmic scale, while they are asymmetric around the mean in linear scale (Figure 7.7). The uncertainty in the estimates is larger than what most people think. Even though the standard curve in the example is based on 7 concentrations of standard covering 6 logs, each measured in triplicates for a total of 21 readings, and the assay has 96% efficiency, the uncertainty in the estimated concentrations of the unknowns is substantial. For example, for “Test 1”, estimated concentration is 46700 copies, with the 95% confidence interval: 31000 – 61000 copies! With less accurate standard curve, the precision in the estimated concentration would be even worse.



	Log(Conc) Low	Log(Conc)	Log(Conc) Up	Repeats	Conc. Low	Conc.	Conc. Up
Test 1	4.4934490538	4.6403340873	4.7872191208	3	31149.354679	43685.175758	61265.942768
Test 2	3.8124309780	3.9494692883	4.0865075985	3	6492.7843427	8901.6248683	12204.151734
Test 3	2.9954390885	3.1494692883	3.3034994881	3	989.55306551	1410.8124655	2011.4048274
Test 4	4.2768800219	4.4181659996	4.5594519773	3	18918.209126	26191.839432	36262.018684
Test 5	4.8034314462	4.9618931616	5.1203548770	3	63596.240975	91599.512357	131933.43718
Test 6	3.3072282896	3.4515399338	3.5958515781	3	2028.7488638	2828.3941731	3943.2251776
Test 7	4.7014554271	4.8556812249	5.0099070228	3	50286.966231	71726.761684	102307.39410
Test 8	5.3415332445	5.5280320167	5.7146307888	3	219549.90047	337312.17479	518239.83075
Test 9	3.9399962614	4.0771184966	4.2142417417	3	8709.5406689	11943.139268	16377.278779
Test 10	5.1137623605	5.2873499216	5.4609374828	3	129945.83394	193798.28152	289026.37955
Test 11	3.5855139776	3.7243779363	3.8632418949	3	3850.4720715	5301.2457380	7298.6391935
Test 12	5.3010503985491	5.4851574734	5.6692645483	3	200009.39609	305602.90163	466943.73021
Test 13	6.9432878254	7.2449625891	7.5466373528	3	8775822.3962	17577721.901	35207675.507
Test 14	5.4682718668	5.6625021750	5.8567324832	3	293948.91864	459729.29095	719005.94817

Figure 7.7 - Top: Calibration by means of the standard curve to estimate the test sample’s concentration and its confidence interval. Bottom: summary of estimated concentrations including confidence intervals in logarithmic (left) and linear (right) scales.

Limit of detection

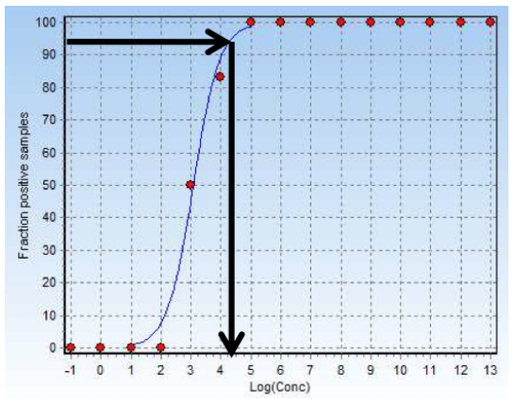


Figure 7.8 - Estimation of LOD by fitting the rate of positive calls as function of concentration

The limit of detection (LOD) is “the lowest amount of analyte in a sample that can be detected with (stated) probability, although perhaps not quantified as an exact value”, with analyte here referring to the targeted nucleic acid (World Health Organization (1995) document WHO- BS/95.173, and EP17-A, Vol 24, no 10 (2004). Approved guideline NCCLS). For classical tests, when a signal is measured against a background, LOD is estimated from the standard deviation of the blank readout at the standard curve intercept. This approach is, however, not applicable to qPCR, which, due to its real-time readout, gives no reading for a negative sample (Cq for a blank sample is formally infinity). Instead, for an analytical process that involves qPCR, LOD can be estimated from multiple standard curves (Burns et al., 2008). A minimum of six is recommended and concentrations around the expected LOD should be assessed. The measured data are transferred to binary format indicating positive and negative PCR’s and the fraction of positive calls at each concentration is calculated. LOD is the concentration at which replicates are positive at the stated rates (e.g. 95 %). GenEx fits the measured positive rates at different concentrations to estimate the LOD (Figure 7.8).

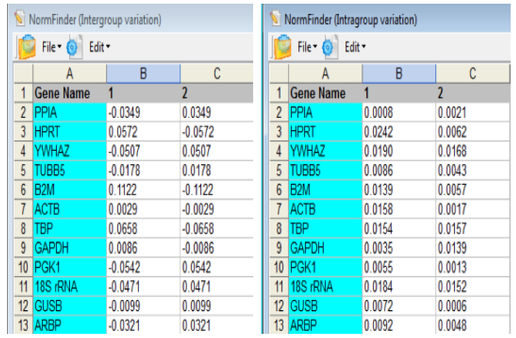
Section 8

Selecting reference genes

With qPCR, the amount of target molecules in a sample is measured rather than their concentration. A large sample is expected to contain more target molecules than a small one and to compensate for the effect of size, normalization must be applied. There are several options as to normalize. A popular option in gene expression analysis is to normalize with reference genes, since this should not only compensate for variation in sample amount, but also for variations in extraction yield, reverse transcription efficiency, and RNA quality. In the early days of PCR, genes needed for basic housekeeping functions were thought to have stable expression and could serve as references. Experience has shown this is not always true, and before a gene is used as a reference, this assumption should be validated. Criteria for a good reference gene is that it has a stable expression among samples and that its expression is invariant of the treatment applied. Stability of expression is reflected by the standard deviation (SD) of biological replicates. However, we cannot just take a set of samples, measure the Cqs and calculate the SD's, because we do not know how to normalize the samples for this exercise. Of course, we can use the same amount of RNA in the analyses, but then, how do we know that all samples were extracted and reverse transcribed with the same yield, and that they have the same mRNA/totalRNA ratio and the same RNA quality? Furthermore, if we were to assume that we can evaluate genes' expression stabilities based on samples normalized to the same amount of RNA, then we would have already decided that total RNA is the best norm. The

gene selected based on minimum SD measured on samples having the same amount of RNA will be the gene that shows a variation that correlates the most with that of total amount of RNA, and we may then as well normalize to the amount of RNA directly. If we suspect that the total amount of RNA is not the best norm, we have to identify optimum reference genes using different strategy.

Figure 7.9 - Estimated intergroup and intragroup variations with Normfinder of genes from the TATAA reference panels in representative brain samples of wild-type and obese mice.



NormFinder (Intergroup variation)

File Edit

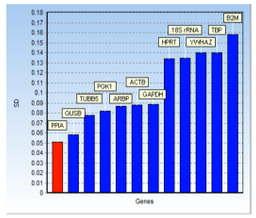
	A	B	C
1	Gene Name	1	2
2	PPIA	-0.0349	0.0349
3	HPRT	0.0572	-0.0572
4	YWHAZ	-0.0507	0.0507
5	TUBB5	-0.0178	0.0178
6	B2M	0.1122	-0.1122
7	ACTB	0.0029	-0.0029
8	TBP	0.0658	-0.0658
9	GAPDH	0.0086	-0.0086
10	PGK1	-0.0542	0.0542
11	18S rRNA	-0.0471	0.0471
12	GUSB	-0.0099	0.0099
13	ARBP	-0.0321	0.0321

NormFinder (Intragroup variation)

File Edit

	A	B	C
1	Gene Name	1	2
2	PPIA	0.0008	0.0021
3	HPRT	0.0242	0.0062
4	YWHAZ	0.0190	0.0168
5	TUBB5	0.0086	0.0043
6	B2M	0.0139	0.0057
7	ACTB	0.0158	0.0017
8	TBP	0.0154	0.0157
9	GAPDH	0.0035	0.0139
10	PGK1	0.0055	0.0013
11	18S rRNA	0.0184	0.0152
12	GUSB	0.0072	0.0006
13	ARBP	0.0092	0.0048

An appropriate approach to select reference genes is a special form of analysis of variance, which in qPCR literature is best known as using the tool Normfinder (Andersen et al., 2004). Normfinder is applied to a panel of candidate reference genes that is analyzed in a set of representative samples. In essence, Normfinder calculates a global average expression of all the genes in all the samples, to which the individual genes are compared. Based on this comparison, SD for each candidate reference gene is estimated. Furthermore, if the samples are from different treatment groups, Normfinder separates the variation into an intragroup and an intergroup contribution. Figure 7.9 shows an example where reference genes were sought for an obesity study in mice, where wild-type mice and an obese strain were compared. The genes were selected from the TATAA reference gene panel, which was measured on seven representative mice from each strain. The intragroup variation estimated is the SD of the genes in the different treatment groups, while the intergroup variation is differential expression and sums to zero for every gene over all the groups. Good reference genes shall have low intergroup variation in all groups and negligible intergroup variation.



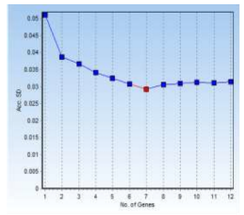


Figure 7.10 - SD (left) and accumulated SD (right) for the reference gene candidates in the TATAA reference gene panel in 14 brain samples from mice estimated using Normfider.

Good strategy using Normfinder is to inspect the calculated intra and intergroup variations to identify any genes that appear regulated or exceedingly unstable and remove them from the data set using the GenEx Data Manager. Normfinder analysis is then repeated without considering the groups, since the remaining genes are not regulated. This produces more

robust result with a single SD estimate for each gene, based on which the genes are ranked (Figure 7.10). The gene with lowest SD is the optimum reference gene. GenEx also calculates the accumulated SD expected if multiple reference genes are used for normalization. If we use larger number of reference genes, random variation among the genes' expression partially cancel reducing the SD. Comparing the SD contributed from different number of reference genes selected based on stability, a minimum in the accumulated SD plot is obtained, indicating the number of reference genes that give the lowest SD (Figure 10). However, analyzing more genes cost time and money, and one should consider the degree of improvement and the overall noise contributed by the reference genes when making a decision. In the example, the largest improvement is observed when including the 2nd reference gene; including additional reference genes only slightly improves the result. Furthermore, the noise contribution from the best reference gene is only 0.05 cycles and as little as 0.04 cycles when combining the two best reference genes. Considering that the repeatability of a qPCR instrument is rarely less than 0.1 cycle (estimated as SD of technical replicates), using more than one reference gene, and definitely more than two, will in this study not improve the quality of the data appreciably.

Using Normfinder, normalization with reference genes can be compared to normalization with total RNA by adding an extra column in the data sheet with the RNA concentrations per analyzed sample in logarithmic scale (Figure 7.11). The algorithm is ignorant of the nature of the variables, and will compare their variation. For the data in our example normalization with total RNA is essentially as stable as normalization with PPIA, which is the single optimum reference gene here. In this study the samples analyzed were flash frozen biopsies from mouse brains, from which RNA of very high quality (RIN 8-9) was extracted. Our experience is that for samples with high quality RNA, normalization to total amount of RNA is often as good as normalizing with a single reference gene. In samples of poor RNA integrity, or when expression may have been induced, normalization with reference genes is preferred.

	PGK1	18S rRNA	GUSB	ARBP	log(RNA)	#group
NC2	21.18	7.61	24.88	20.49	2	1
NC3	21.07	7.72	24.83	20.32	2	1
NC4	21.24	7.78	25.03	20.62	2	1
NC5	21.48	7.7	25.12	20.6	2	1
NC6	21.29	7.68	25	20.55	2	1
NC7	21.13	7.7	24.97	20.49	2	1
HFD1	21.38	7.58	24.92	20.7	2	2
HFD2	21.28	7.66	24.97	20.64	2	2
HFD3	21.29	7.63	24.94	20.6	2	2
HFD4	21.41	7.98	25	20.65	2	2
HFD5	21.25	7.8	24.96	20.57	2	2
HFD6	21.27	7.78	24.91	20.51	2	2
HFD7	21.37	7.79	24.99	20.51	2	2

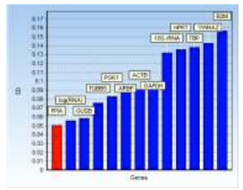


Figure 7.11 - Left: Input data for comparison of normalization with reference genes to normalization with total amount of RNA using Normfider. A column indexing the used total RNA concentrations in logarithmic scale has been added. Right: Output indicating that for these particular samples total RNA normalization is almost as stable as normalization with the single optimum reference gene.

An older method to identify good reference genes that still is being used is geNorm. It uses the same input data as Normfinder, but it does not consider groups; all samples are treated as being from a single population. geNorm sequentially eliminates the gene that shows the highest variation relative to all the other genes based on paired expression values in all the studied samples. The variability is reflected by a so called M-value (Figure 7.12). Because of the elimination process, geNorm cannot identify an optimum reference gene, and ends up by suggesting a pair of genes that shows high correlation and should be suitable for normalization. The M-value is related to the SD, but as calculated, the M-values for the genes are based on different sample sizes and are therefore not strictly comparable. Furthermore, as the comparison of any individual candidate gene is performed toward a plurality of genes, assumed to resemble most closely the anticipated stable behavior, it is prone to systematic failure where group of co-regulated instable genes may be involved in the analysis. Any such co-regulated complex of instable genes may dominate over the stable genes and hence point at deviant genes as candidates. Usually, the gene rankings by geNorm and Normfinder are similar, which is reassuring. Should the rankings differ, there would be a reason to suspect the selection to include one or more regulated genes, and the result should be interpreted with caution.

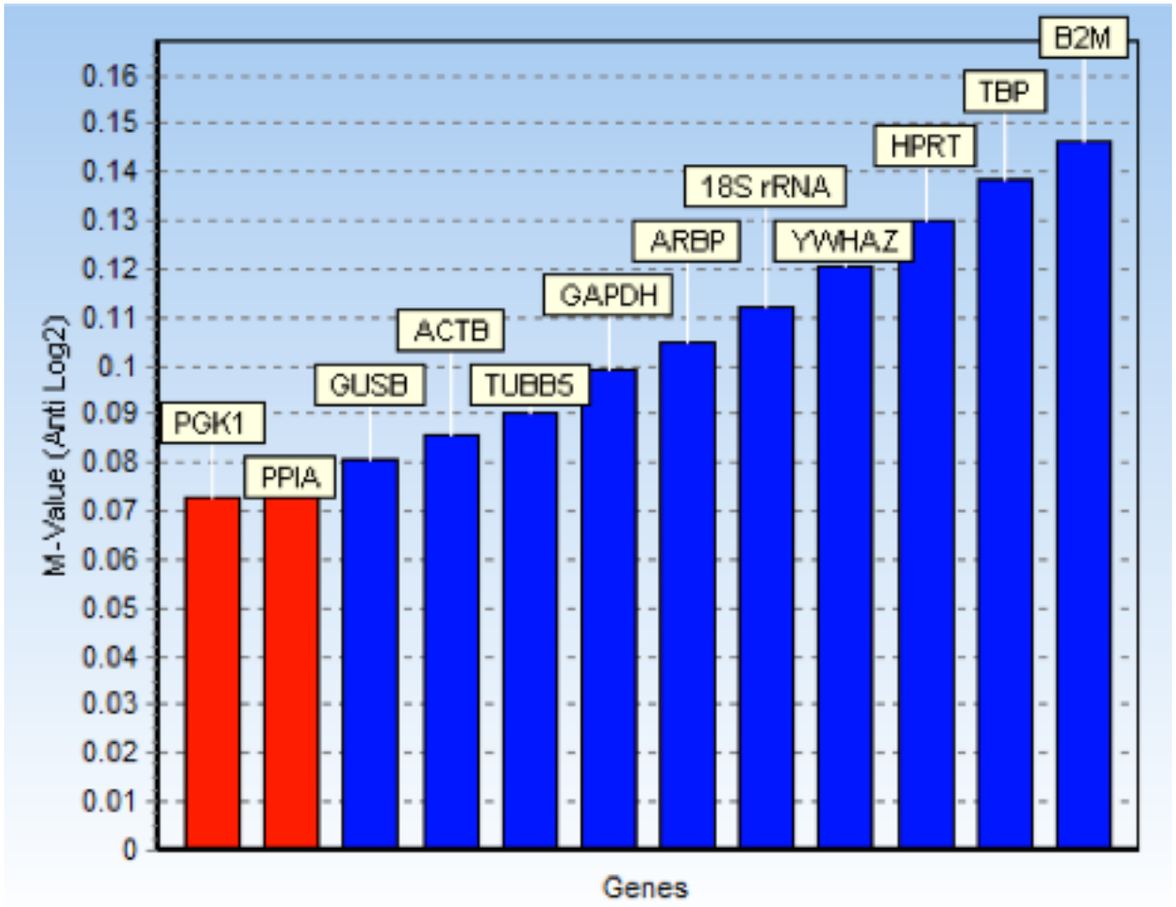


Figure 7.12 - Ranking of reference gene candidates be sequential elimination using geNorm. The final two genes selected cannot be compared further.

Section 9

Relative quantification

Treatment groups are readily compared visually in bar graphs using descriptive statistics (Figure 7.13) and statistical comparison is made using ANOVA (one factor, two or more levels) or 2-way ANOVA (two factors, two or more levels each) or, in the case of two groups, with either t-test (paired/unpaired, 1-tail/2-tail) or non-parametric tests (Mann-Whitney, Wilcoxon). The difference between the groups is shown in either linear or logarithmic scale and the confidence interval is indicated (Figure 7.14). Note that the confidence interval of the differential expression is asymmetric when data are presented in linear scale. When expression of many genes is compared, GenEx offers several means to control for the false discovery rate due to multiple testing, including Bonferroni, Benjamini-Hochberg, Westfall & Young, and Benjamini-Yekutieli (Figure 7.15).

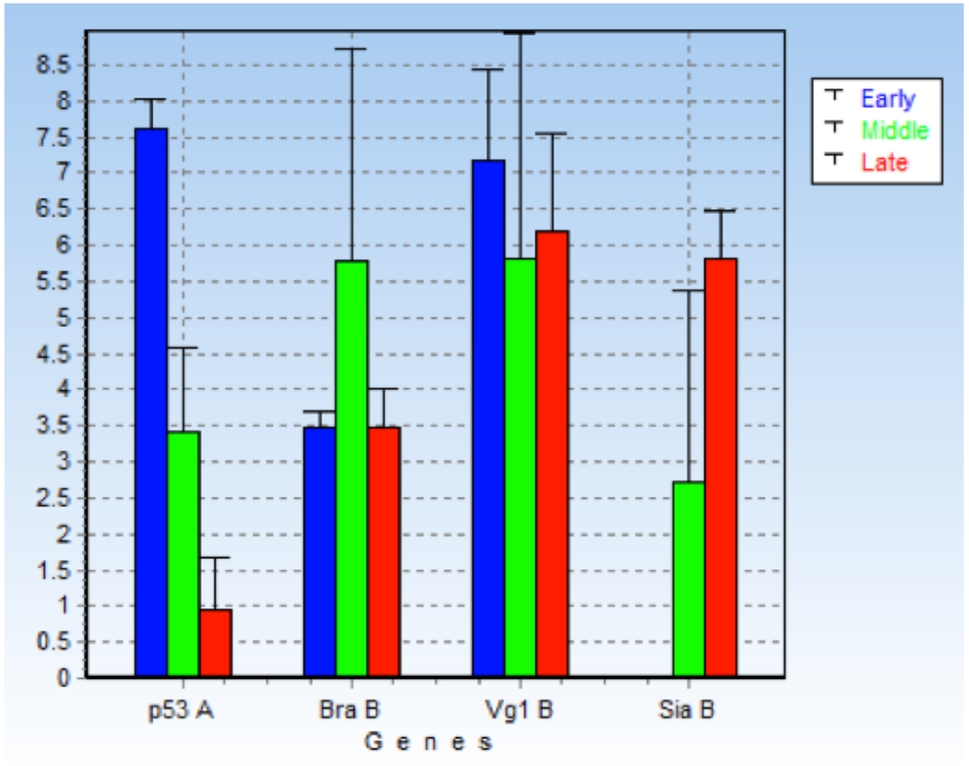


Figure 7.13 - Comparison of the expression of four genes in three groups. Bars indicate mean expression and the error bar either SD, SE or CI.

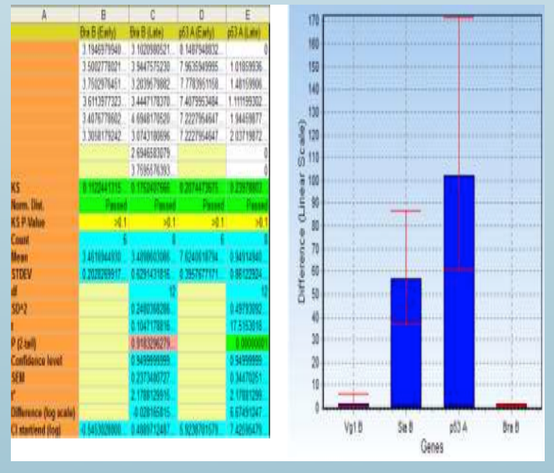


Figure 7.14 - Comparison with t-test of the expression of four genes between two groups. Left: Descriptive statistics includes normality test and p-values. Right: Bar graph showing the differential expression in linear scale. Note, the indicated confidence intervals are asymmetric around the means.

	KS Group 1 (...	KS Group 2 (...	Difference bet...	P Value	P (Benjamini-Hoc...	P (Westfall & Young)	P (Benjamini-Yekutieli)
RG4	Passed	Passed	-1.18826398	0.00005062	0.0011147	0.00257	0.0051251
RG17	Passed	Passed	-1.10118023	0.000118	0.00127812	0.00494	0.00587647
RG8	Passed	Passed	-1.36187953	0.00012155	0.00127812	0.00494	0.00587647
RG7	Passed	Passed	-1.18182055	0.00031428	0.00150089	0.01037	0.00690071
RG2	Passed	Passed	-1.49825382	0.00036413	0.00150089	0.01081	0.00690071
RG10	Passed	Passed	-1.22262582	0.00052804	0.00197747	0.01433	0.00909189
RG11	Passed	Passed	-1.12882321	0.00126256	0.00368418	0.02837	0.01693892
RG19	Passed	Passed	-0.97831093	0.00218714	0.00498312	0.04268	0.02291108
RG1	Failed	Passed	-1.08236183	0.0022424	0.00498312	0.04268	0.02291108
RG16	Passed	Passed	-0.90007345	0.0025065	0.0050130	0.04268	0.02304884
RG12	Passed	Passed	-0.83237989	0.00286956	0.00522101	0.04268	0.02400484
RG5	Passed	Passed	-1.0327149	0.00303341	0.00522101	0.04268	0.02400484
RG14	Passed	Passed	-1.04575987	0.00407095	0.00635184	0.04449	0.02920409
RG3	Failed	Passed	-0.873477	0.00447486	0.00639266	0.04449	0.02939178
RG20	Passed	Passed	-1.05784231	0.00701563	0.00969331	0.05364	0.04456732
RG15	Passed	Passed	-0.61494415	0.00814714	0.01029238	0.05364	0.04732171
RG18	Passed	Passed	-0.76938131	0.00932495	0.01145255	0.05364	0.05265586
RG6	Passed	Passed	-0.73924194	0.01954786	0.0234691	0.06705	0.10790482
RG9	Passed	Passed	-0.77903746	0.04688727	0.04976569	0.09908	0.22880968
RG13	Passed	Passed	-0.48639622	0.20140296	0.20206905	0.20406	0.92906087

Figure 7.15 - Comparison of the expression of multiple genes between two group corrected for the high false discovery rate due to multiple testing. Columns 2 and 3 indicate result of normality test and column 4 the differential expression in log scale. Colum 5 indicates p-values calculated with t- test. Green indicates p-values considered significant based on Bonferroni correction, yellow are p- values below stipulated uncorrected confidence level that are not significant with Bonferroni correction and red are p-values above confidence threshold. The three last columns indicate p- values calculated using Benjamini-Hochberg, Westfall & Young, and Benjamini-Yekutieli corrections for the high false discovery rate.

Expression profiling

T-test and ANOVA are univariate methods that analyze the expression of every gene separately, effectively assuming that the genes are expressed independently of each other. This is rarely the case; genes' expressions tend to be correlated. This correlation can be exploited in the analyses using multivariate statistical methods. GenEx offers several unsupervised as well as a selection of supervised methods to classify samples and categorize genes based on expression profiles. Unsupervised methods classify samples and genes based on the measured profiles only. They include classical hierarchical clustering combined with heat map, which can be based on various clustering schemes including the Ward's algorithm and several distance measures including the Euclidian distance and the magnitude of the Pearson correlation. While the Euclidian distance clusters genes based on similarities and consider up-regulation and down-regulation being opposite, hence anticorrelated, distance based on the magnitude of the Pearson correlation considers up-regulation and down- regulation to be correlated. The latter is useful to classify, for example, genes that show the same temporal response to treatment independently of the genes being up or down-regulated. The clustering is visualized in a dendrogram, which in GenEx can be mirrored in every node. Mirroring in a node changes the visual appearance of the dendrogram, producing an equivalent mathematical solution. A small Self Organized Map (SOM) can be used to force classification into a defined small number of groups based on expression similarities. SOM can also be used to validate a classification model,

based on the distribution of samples/genes in a large map. Principal Component Analysis (PCA) groups samples/genes based on correlated expression in reduced space. Figure 7.16 shows example of hierarchical clustering, SOM, and PCA of genes expressed during the development of the African claw frog Xenopus Laevis from the oocyte to tadpole stage (Bergkvist et al., 2010). Hierarchical clustering of sample and genes can be combined showing also the measured intensities in a heat map (Figure 7.17). The appearance of the heat map can be changed in GenEx within equivalent mathematical solutions by mirroring the dendrograms' in nodes.

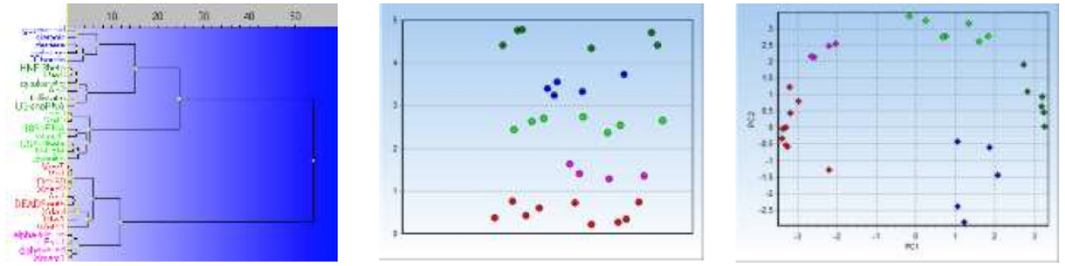


Figure 7.16 - Classification of genes expressed during developmental of Xenopus laevis based on hierarchical clustering (left), SOM (center), and PCA (right). The dendrogram can be mirrored in any of the white nodes.

Supervised methods require a training set of samples with known classification; for example, negative and positive samples, or short, medium and long term survivals. A model is developed based on the training set that can be used to classify new data (in GenEx called test data). The procedure is similar to a regression based on standard curve, but here it is based on multiple genes and the model does not have to be linear. Supervised methods available in GenEx include Partial Least Squares (PLA), which is used to calculate a single standard curve based on the expression of multiple genes to predict concentrations or other measures of test samples. Potential Curves is a variant for prediction of new data based on PCA, and Artificial Neural Network (ANN) and Support Vector Machines (SVM) are multivariate non-linear methods to classify samples. Logistic regression, Probit, Receiver Operating Characteristics (ROC), and Survival Analysis will soon also be available.

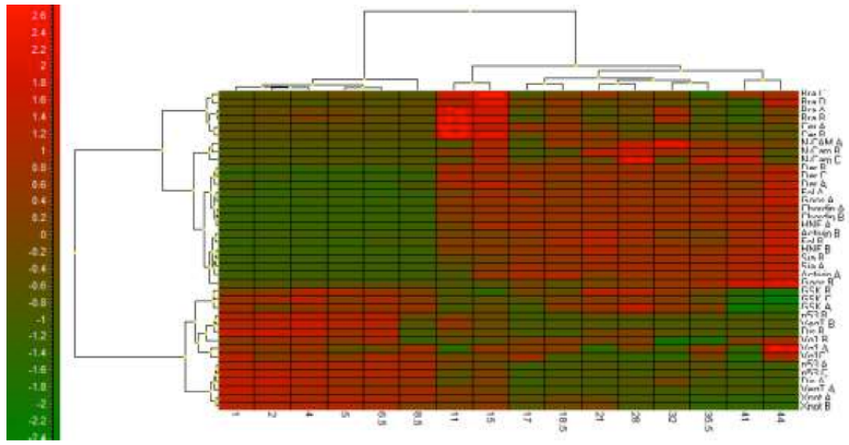


Figure 7.17 - Classification of genes and samples visualized with dendrograms and a heat map indicating expression levels. The heat map appearance can be altered by mirroring in any of the dendrograms' nodes.

Experimental design

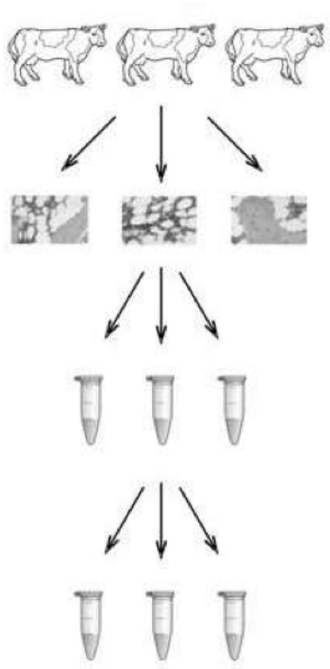


Figure 7.18 - Nested experimental design. Three subjects (heifers) are tested. Three samples are collected from each subject and extracted. The extracted material is reverse transcribed in triplicates. Each cDNA is analyzed in triplicates for qPCR.

Designing experiments is more difficult than analyzing results. In a well designed experiment, confounding variation is minimized and the number of subject is sufficient to obtain conclusive results. A good strategy is to perform a fully nested pilot study before specifying the test protocol for a larger study (Figure 7.18) (Tichopad et al., 2009). Figure 7.19 shows the result of a nested pilot study wherein three heifers were studied by collecting three blood samples of each that were reverse transcribed in triplicates, and each cDNA was analyzed in triplicate using qPCR. Using a nested ANOVA, the variation arising from the different experimental steps can be estimated and expressed either as standard deviations or as variance contributions (Kitchen et al., 2010). While β-actine and Caspase-3 show generally low standard deviations in all steps, Interleukin 1- β and Interferon-γ levels varied substantially among the heifers.

For liver samples, the picture was different, with the data evidencing large variation in the sampling step. Knowing the costs associated with the different experimental steps, the follow up study can be cost optimized. For example, for genes exhibiting SD = 0.1 cycle for the qPCR and RT steps, SD = 0.2 cycles for the sampling/extraction step, and SD = 1 cycle for the variation among the animals, and assuming a cost of 1 unit for the qPCR, 3 units for the RT, 10 units for sampling/extraction, and 100 unit for each animal, with a total budget of 1000

units, the best we can do is to analyze 8 animals, sample each animal once, perform RT in triplicates and qPCR in duplicates.

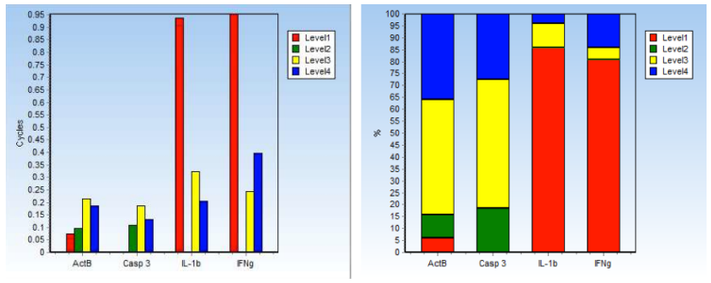


Figure 7.19 - Decomposition of the variance in a 4-level nested study of blood samples collected from heifers. Left: estimated standard deviations (SD) among heifers (red), among replicate blood samplings (green), among reverse transcription replicates (yellow), and among qPCR replicates (blue). Right: Same data presented as variance contributions expressed in percentages.

The total standard error (SE) for this study is expected to be about 0.36 cycles (Figure 20). Using the same tool, the SD among animals analyzed with a single sample collected from each animal, RT performed in triplicates and qPCR in duplicate is estimated to 1.02 cycles. This can be fed into a Power analysis to estimate the number of animals needed to ensure a particular difference with certain confidence and power. If we accept 5 % false positive rate (95 % confidence) and 5 % false negative rate (95% power) we construct a graph showing how many subject are needed to measure a particular difference due to treatment, For example, to measure a 2-fold difference (ΔCq of 1) we require under these criteria 15 animals (Figure 7.20).

	Level 1	Level 2	Level 3	Level 4	Tot. Var.	SD	Cost
Design Total	8	1	3	2	0.130625	0.3614208073...	1000
Design Subject	1	1	3	2	1.045	1.0222524150...	125

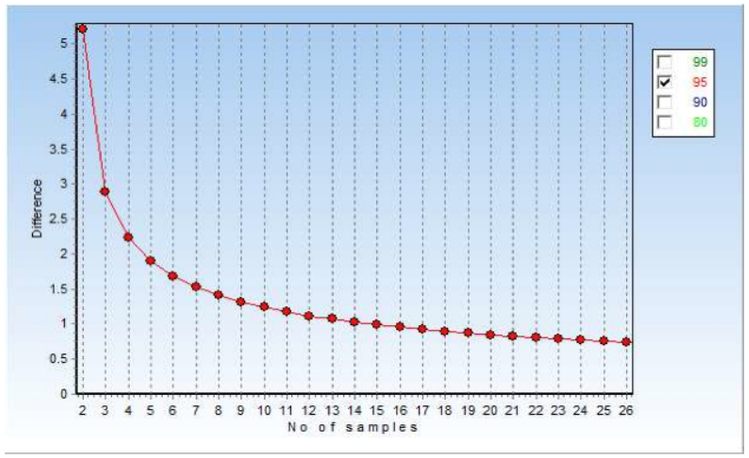


Figure 7.20 - Power analysis assuming SD=1.055 cycles and 5% false positive and 5 % false negative calls. Straight line and arrow indicates that 15 subjects are needed to measure a difference of 1 cycle due to treatment under those conditions.

Literature

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World Health Organization.

Chapter 14

Improved data reproducibility using MIQE-compliant, automated qPCR workflows

Practical guide on how to successfully use MIQE guidelines

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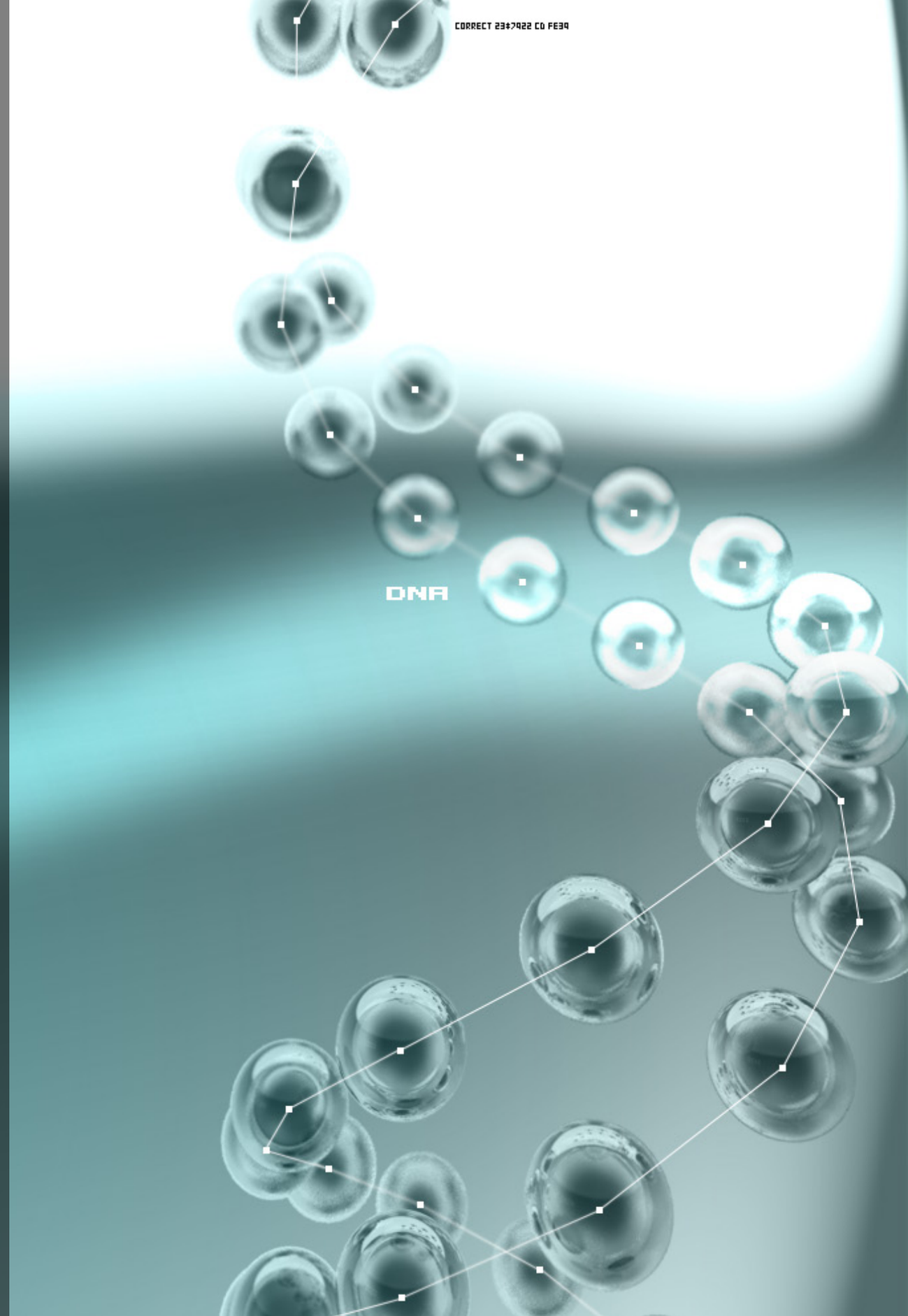


Section 1

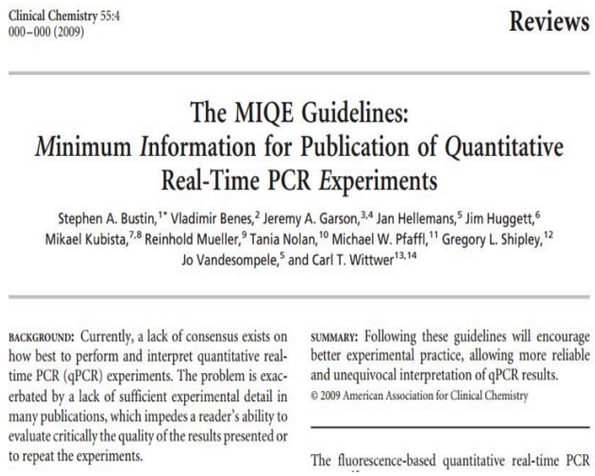
Introduction

Quantitative PCR (qPCR), (also known as real-time PCR) is one of the most widely used molecular biology techniques in basic research, quality control operations, diagnostic applications, and RNA/DNA quantification services. qPCR can be used as a standalone method or in combination with other techniques, such as next generation sequencing (“NGS”) and gene expression technologies (DNA microarrays), for which it is generally used as a supporting quality- and quantity-control tool.

Figure 8.1 - Working with automation

qPCR was one of the first “big data” molecular biology techniques, as the large volumes of data produced are not simple machine readouts or DNA fragment images, and cannot be directly or quickly interpreted by laboratory personnel. To understand qPCR data, appropriate data analysis methods must be applied. Compounding the complication, qPCR results are highly dependent on several user-controlled parameters, such as the quality of the experimental design, wet-lab performance, process quality control, and data analysis. Due to the high sensitivity of qPCR assays, even the slightest contamination of samples, reagents or equipment can degrade the quality of the results. Minor mistakes can be expensive – repeating experiments requires additional time and reagents, which are expensive, and samples, which may be difficult or impossible to replace. To ensure reliable and accurate results, qPCR was one of the first techniques that required users to undergo specific training to master the various steps, including study and experiment design, good and clean laboratory practice and precision pipetting as well as “dry lab” data analysis and quantification protocols.

It is expected that errors will be introduced by inexperienced users who are at the beginning of their scientific careers, such as undergraduate students or researchers new to pipetting small volumes. However, even researchers who have considerable experience in qPCR can produce poor results. Laboratories in which qPCR is not the main technique for detection and quantification of nucleic acids, but rather a supporting technique, are particularly vulnerable. Users in such laboratories might perform qPCR occasionally for validation of DNA microarrays or preparation of libraries for NGS. Often multiple users are involved in a research experiment, introducing inter-operator variability and other sources of variation that can affect the end results. These issues are particularly problematic in regulated environments.



Clinical Chemistry 55:4
000–000 (2009)

Reviews

The MIQE Guidelines:
*M*inimum *I*nformation for Publication of Quantitative
Real-Time PCR *E*xperiments

Stephen A. Bustin,[1*] Vladimir Benes,[2] Jeremy A. Garson,[3,4] Jan Hellemans,[5] Jim Huggett,[6]
Mikael Kubista,[7,8] Reinhold Mueller,[9] Tania Nolan,[10] Michael W. Pfaffl,[11] Gregory L. Shipley,[12]
Jo Vandesompele,[5] and Carl T. Wittwer[13,14]

BACKGROUND: Currently, a lack of consensus exists on how best to perform and interpret quantitative real-time PCR (qPCR) experiments. The problem is exacerbated by a lack of sufficient experimental detail in many publications, which impedes a reader's ability to evaluate critically the quality of the results presented or to repeat the experiments.

SUMMARY: Following these guidelines will encourage better experimental practice, allowing more reliable and unequivocal interpretation of qPCR results.

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The fluorescence-based quantitative real-time PCR

Figure 8.2 - The MIQE Guidelines publication

In this chapter we highlight why and how automation — specifically advanced liquid handling and information management software — improves traceability, complies with Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009) and standardization, and eliminates or decreases inter-operator variability. All this is becoming increasingly important even in non-

regulated scientific research as it leads to enhanced reproducibility of scientific discoveries, which has recently become a new initiative of the United States National Institutes of Health (NIH), one of the world's argest grant funding organizations (Collins et al., 2014; Principles and Guidelines for Reporting Preclinical Research). We also discuss certain conventional practices, point out the differences between good and poor methods, and offer solutions.

Standardization of workflows

The first organizations requiring standardized qPCR workflows based on internal needs or external regulations were industrial and diagnostic laboratories performing routine operations.. Such workflows must accurately define each step (e.g, sample preparation, nucleic acid extraction, assay design, experimental conditions, and data analysis), and must include a standard operating procedure or similar document. Additionally, each step requires quality control checks, allowing the user to determine whether the workflow is proceeding properly or to describe any errors that may have occurred during the procedure. To eliminate contamination and ensure accurate and reproducible results, the laboratory must invest in reliable equipment, regular maintenance and validation plans for the equipment, and proper working conditions (e.g., clean environment and processes that regulate a one-way flow of samples).

“Standardization of workflows can reduce errors but cannot eliminate them.”

However, even under optimal laboratory conditions, qPCR results are heavily dependent on many factors relating to the reaction, including amplification efficiency of primer–probe combinations, the physical properties of target DNA or RNA regions, the quality of the nucleic acid extraction protocol, the sample

matrices and the cycling conditions. Some assays perform differently with different combinations of nucleic acid extraction protocols, sample matrices or cycling conditions. To reduce user-introduced error, industrial and diagnostic laboratories must validate their own qPCR assays or use validated commercial diagnostic kits (which shorten the validation process drastically) and use welldefined workflows and reagents.

The use of commercial kits is less common in basic research and non-human diagnostic laboratories, because they are not readily available (kit manufacturers emphasize human diagnostics).). Researchers in these labs also tend to distrust commercial kits that do not completely disclose reagent composition, target information, and assay validation results. As an alternative, researchers often must design and validate qPCR assays themselves or use those methods found in the scientific literature. The scientific freedom to design individual assays and experiments results in non-standardized workflows. To address this, a growing number of researchers are using MIQE guidelines to standardize their qPCR workflows with the purpose of increasing the reliability of published qPCR data, while maintaining experimental freedom. Even with this level of standardization, variation between samples can still occur, especially when different types of samples or matrices are used.

Validation of entire qPCR workflows for diagnostic applications requires more elaborate principles than those provided by the MIQE guidelines, and must take into account regulations for the specific diagnostic field, e.g. genetically modified organisms or medical molecular diagnostics (Broeders et al., 2014; Raymaekers et al., 2011; Wallace et al., 2013; Zel et al., 2008). In certain cases, inter-laboratory tests (ring tests) should be performed to ensure that the execution of the assay in different laboratories give consistent results, meaning that the assay is reliable and robust.

Standardization of workflows can reduce errors but cannot eliminate them. Increased traceability enables laboratories — whether academic, industrial or regulated — to detect errors, providing a basis for preventive and/or corrective measures and thus complementing standardization efforts.

Section 3

Traceability

Assuring traceability of test samples with their results is one of the biggest challenges faced by industrial laboratories, particularly in diagnostics. Traceability is essential for service providers working in regulated environments, such as those under International Organization for Standardization (ISO) and Good Laboratory Practice (GLP). In these laboratories, it is frequently necessary to locate data for a particular sample that may have been analysed months or years ago. It is necessary to record and track every assay parameter, reagent, and piece of equipment used to verify the sample identity with its corresponding results. Such tracking requires superb traceability and is usually enforced by complex quality-assurance systems. Unfortunately, quality-assurance systems, which are generally very time consuming and expensive to implement and maintain, can become an independent source of errors. Eventually, after the data are recorded and stored, there is still a system requirement for retrieving desired information as quickly as possible.

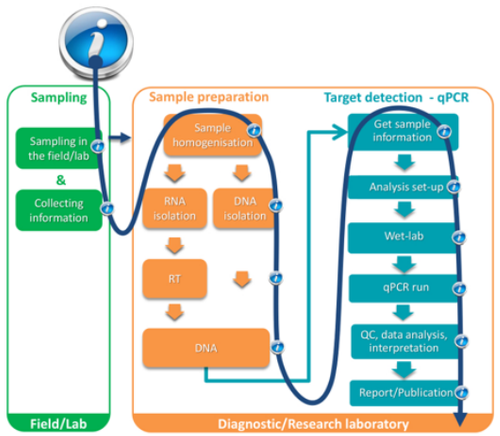


Figure 8.3 - Traceability means collecting and documenting information at each step through the qPCR workflow.

“Organizations that provide research grants and scientific journals that publish peer-reviewed articles consider traceability increasingly essential for ensuring the quality and reproducibility of results.”

Scientists in basic research environments that do not require such stringent regulatory requirements are understandably less willing to take on these additional steps to ensure accurate sample traceability. Usually the entire traceability of the sample from the beginning of the experiment to the end depends on the willingness of an individual researcher to diligently collect and record all necessary information in laboratory notebooks. Of course, laboratory notebooks do not offer a systematic method for collecting and recording relevant information and distinguishing it from irrelevant data, and when this task becomes tedious, relevant information can be omitted. In addition, finding information in print laboratory notebooks can be time consuming.

However, organizations that provide research grants and scientific journals that publish peer-reviewed articles consider traceability as increasingly essential for ensuring the quality and reproducibility of results (Collins et al., 2014; McNutt, 2014).

Modern molecular biology techniques, starting with qPCR, are much more complex than their predecessors, particularly with respect to documenting and analysing results. The extensive raw data produced by qPCR requires expansive data-storage capacity. Standard rules for data analysis do not currently exist, and, depending on the scientific goal, various methods can be applied to the same set of data, each using different data analysis protocols and parameters. It is imperative that the sample identity, experimental method, data analysis, and corresponding results are stored together for easy retrieval in the event the experiment needs to be repeated or verified at a later date.

As emphasized here, it is also important to balance the need for good laboratory practice with the need for scientific freedom that allows creativity. Tools are clearly required to help scientists in all laboratories cope with the demand for sample traceability when using these modern methods and the immense volumes of data that they produce.

Section 4

Inter-operator variability and transfer of knowledge within institutions

Unlike industrial, diagnostic and service environments, which are all highly regulated, standard operating procedures do not exist for non-regulated laboratories when designing qPCR assays, performing experiments, or analysing data.. Generally, any guidelines that are applied are usually non obligatory. Researchers often use laboratory-specific methods to obtain results and, due to the individual and focused nature of the research, may not need to share them with colleagues.. Protocols are frequently adapted from published work, including improvements developed during previous projects, and passed on from one work group to the next. Many published protocols are difficult to reproduce due to the lack of experimental details in the publication. Similarly, protocols that are passed from one researcher to the next as laboratory notebook entries may require user interpretation that can result in large inter-operator variability.

In laboratories where the same person performs and diligently documents all the experiments for a particular project, inter-operator variability is not an important concern. However, it becomes increasingly important in laboratories with high turnover rates for students and technicians as well as large, multi-scientist projects. Information can be lost when transferring data from one worker to the next: handwritten notes can be difficult to read, and data can be difficult to find if the experiment date is not known or many notebooks are involved.

Slider 8.1 - Information to store in the laboratory related to qPCR in order to improve traceability

Unlike regulated industrial and diagnostic environments, where the protocols must be validated prior to use and executed in the same way by different operators, standardization of methods in unregulated laboratories are allowed to happen gradually if at all. In an ongoing effort to standardize workflows and minimize inter-operator variability, it is necessary to transfer protocols to standard operating procedures (SOPs) and rigorously monitor changes with forms, checklists, and signatures. The increased requirements for sample traceability will demand additional resources and solutions that ensure traceability without corresponding increases in user workloads. Software solutions such as electronic laboratory notebooks (ELNs) or laboratory information management systems (LIMS) are often used to implement traceability, however even these systems are not ideal as we will discuss later in the chapter (Machina et al., 2013a; Machina et al., 2013b).

Section 5

Applying MIQE guidelines to automate qPCR workflows

APPLYING THE MIQE GUIDELINES TO AUTOMATE QPCR WORKFLOWS

1. Nomenclature
2. Sample preparation
3. Nucleic acid extraction
4. qPCR protocol
5. Data analysis
6. Other data relevant to qPCR experiments

To address problems that arise from lack of documentation of qPCR experiments, Stephen Bustin et al. (2009) published the MIQE guidelines in a novel attempt to encourage scientists all over the world to perform and publish qPCR experiments in a standardized way. The concise and well-conceived guidelines details how qPCR experiments should be designed, performed, and documented by all researchers to increase confidence in the results.

In this section, we present the parallel flows of information required for method development and physical processing of the samples (e.g., DNA, RNA) during qPCR and how these flows are addressed in automated work solutions for qPCR experiments.

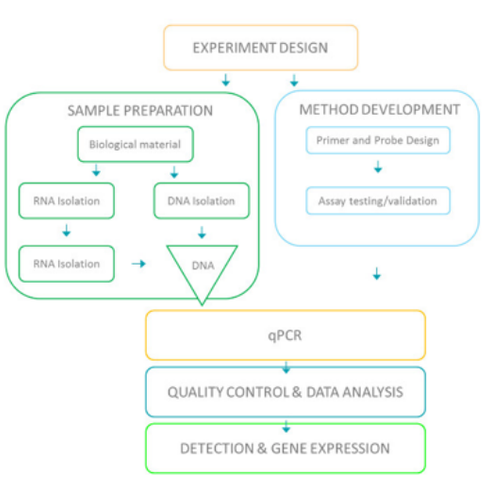


Figure 8.4 - An example of a qPCR workflow

Previously, laboratory automation was limited to liquid handling instruments (“pipetting robots”) that transferred liquids in routine applications. As the software became more advanced, more sophisticated instruments have been developed that incorporate standardized workflows, implement traceability, and minimize inter-operator variability.

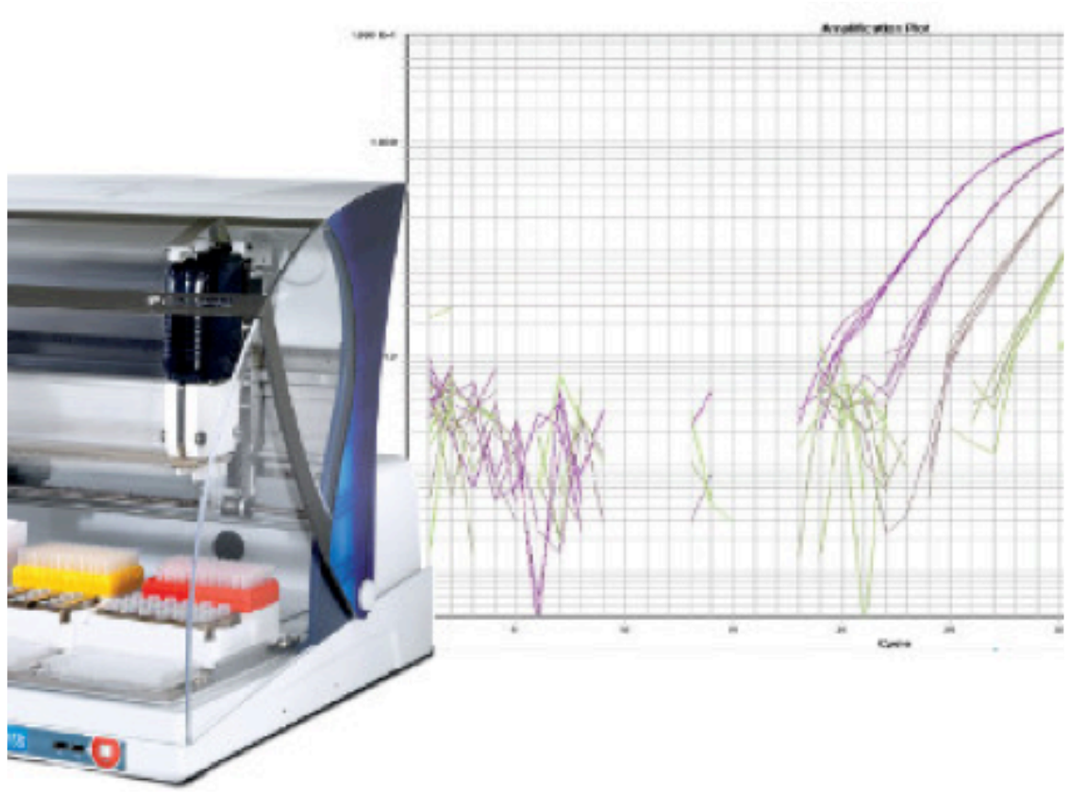
Automation of qPCR experiments involves two key sources of information management: 1)experiment-specific information, including sample source, assay reagents, reaction mixture compositions, QC controls, cycling conditions, qPCR cyclers, etc. and 2)liquid-handling information including pipetting tasks, lab ware templates, volume restrictions, and general liquid transfer methods (i.e., sample dilutions, reagent preparations, and combining samples with reagents).

Available solutions for both information requirements vary with respect to sophistication and appropriateness for direct application to qPCR. Several groups developed or customised LIMS and ELNs to address these issues. These systems are often limited to specific research domains and types of data (e.g., genotyping, protein production, protein–protein interaction, two-dimensional gel electrophoresis) and target-specific laboratory tasks (e.g., sample management, laboratory workflows and protocols, documentation) and are not well integrated with liquid handling equipment. A list of existing LIMS and ELN solutions is available on this link.

The expanding qPCR market has encouraged solution providers to develop additional and specific tools for qPCR:

- For experiment-specific information: PCR or qPCR-specific plugins are now available for ELNs, and qPCR-specific ELNs have been developed (see Slider 8.2).

Slider 8.2 - PCR- and qPCR-specific software modules developed for liquid-handling instruments



Gilson Pipetmax

• For liquid-handling information: PCR- and qPCR-specific software modules developed for liquid-handling instruments make it increasingly easy to execute qPCR experiments. Available systems vary with respect to flexibility, qPCR-specific documentation and traceability, and ease of integration into the information flow (see Figure 8.5 and Table 8.2).

Recently, attempts have been made to merge the experiment-specific information with the liquid handling aspects of qPCR automation. There is a clear tendency towards automated liquid-handling instruments that provide an increasing number of information management functions, such as a qPCR assay repository, the ability to create flexible, new versions of singleplex or multiplex qPCR assays, generation of print-ready reports detailing the qPCR run setup, and integration with external devices, such as qPCR cyclers. These developments offer valuable aides for standardization and traceability and indicate that a growing number of MIQE requirements are being automated.

Here we discuss the nine sections of the MIQE guidelines that are relevant for automation.

Manufacturer	Automated Liquid Handler/ Software
Gilson	PIPETMAX qPCR Assistant
Hamilton	PCR setup Workstation for STAR and NIMBUS platforms
Tecan	Freedom EVO PCR workstation with TouchTools™ PCR Wizard
Eppendorf	epMotion with epBlue software
QIAGEN	QIAgility
Agilent Technologies	Bravo
Perkin Elmer	JANUS® Automated Workstation
Beckman Coulter Lifesciences	Biomek 4000

Table 8.2 - Examples of liquid handling instruments with PCR- and qPCR-specific software modules

Figure 8.5 - GENEIO, a qPCR specific ELN connects entire qPCR workflow

Nomenclature

The first task undertaken for the standardization of qPCR was to develop a certain nomenclature ensuring that workflows are easy to understand for a wide audience and that terms and abbreviations, such as “Cq” for quantification cycle, are unambiguous. All automated qPCR solutions should adopt this nomenclature if they have not already done so.

Sample preparation

Information relating to sample preparation , including where and how the samples were obtained, stored, and prepared, is very important and should be properly recorded, particularly when different types of samples (e.g., different tissues) from the same organism are being tested. Additionally, samples of the same origin may require different experimental approaches, such as the preparation of serial dilutions, the details of which should also be recorded.

Sample preparation information should be recorded in lab notebooks or into a LIMS/ELN system immediately after each step to reduce the risk of losing the information. Ideally, this information should be recorded at the same time and location as the sample acquisition. Obtaining samples from the field presents special challenges to the information recording process.

When using automated solutions, the information flow through different parts of the qPCR workflow, such as sample preparation and automated liquid handling, should be integrated to ensure traceability. Using unique sample names or sample containers (e.g., microcentrifuge tubes) that are clearly identified using, for example, a barcode, can facilitate sample documentation. Additional descriptors can be added to samples such as dates of DNA/RNA isolation, source of sample, organism, tissue type and similar.

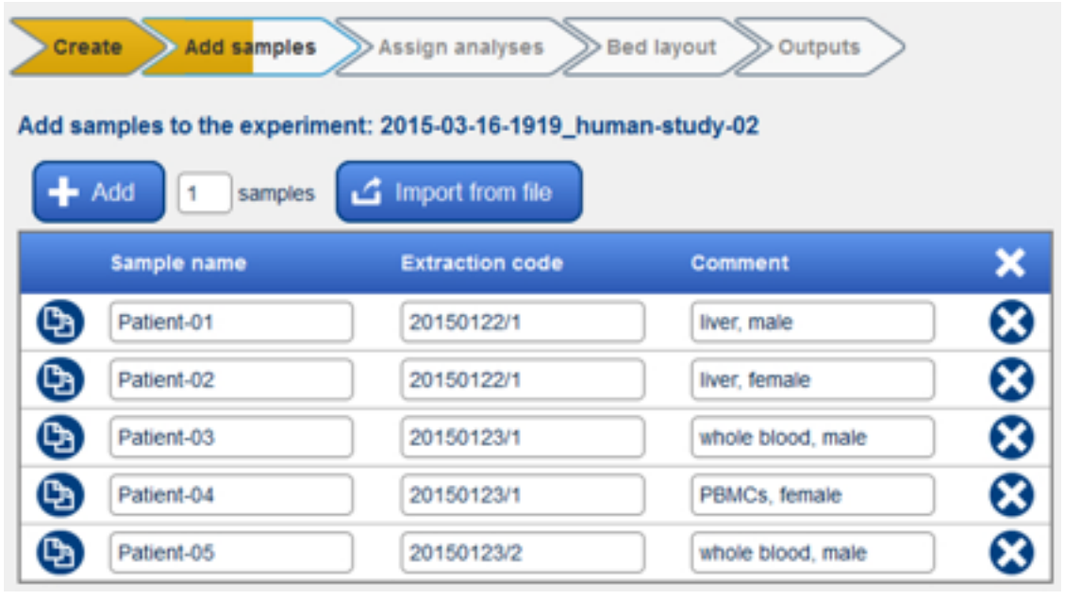


Figure 8.8 - Example of additional information linked to samples that can be stored to increase traceability in liquid handling automation software

Nucleic acid extraction

In most qPCR laboratories, RNA or DNA extractions from crude sample preparations are performed manually or with dedicated automated instruments using columns or magnetic-beads. In some cases, liquid handling instruments with specific nucleic acid extraction add-ons can be used. Nucleic acid extraction instruments are typically standalone devices and have very limited input and output capabilities. Generally, information management for traceability purposes depends entirely on the users and poses a possible source of error. Regardless of the number of instruments used for nucleic acid extraction and QC methods and where the corresponding details are stored, it is essential that information about the protocols used for preparing each sample can be easily accessed.

Nucleic acid quality control (QC), usually an integral part of nucleic acid extraction, is also typically performed on dedicated instruments. QC results should be linked to the original sample names.

Advanced automated liquid-handling instruments that do not include nucleic acid extraction and QC steps can integrate such information (for example, as a user-input comment) or, at least, link liquid handling information to samples, increasing the traceability.

Fully integrated systems for automated liquid handling document workflows through nucleic acid extraction, QC and raw Cq results. These systems are typically tailored to a specific and completely standardized qPCR application, such as human forensics. These dedicated systems are too expensive for most research labs, in which qPCR setups vary from run to run.

qPCR protocol

The MIQE standard requires that several parameters related to processes prior to the qPCR assay are recorded in the following MIQE steps: qPCR target information, qPCR oligonucleotides and qPCR validation.

Conventional automation approaches (automated liquid handling or software management systems) typically do not document these steps. Individual experiments (e.g., determining limit of detection) can be executed as separate qPCR experiments, and users are often required to manage and document these steps manually in external resources. As in other aspects, industrial laboratories and regulated environments typically provide more consistent documentation procedures than do research laboratories. However, documentation of these qPCR protocol steps is not currently as well supported as other steps of the workflow. Additional software management systems will likely be

Exp0012_2014-01-22_BIO_HAV+NOVwater

Experiment created on: **22.01.2014** by: **Katarina Kovac (kkovac)**

Mastermix Recipes

Analysis: HAV+NOV_detection_ABI7900_384-v1

Mastermix 1: **HAV**

Required No. of reactions: **41**

Extra mastermix volume: **5.0%**

44 reactions

Reagent	Reagent ID	Initial conc.	Final conc. / reaction	Number of reactions	
				1	44
water		-	-	0.8	**35.2**
Master Mix		2.0 x	1.0 x	5.0	**220.0**
Primer Fw		200.0 µM	18.0 µM	0.9	**39.6**
Primer Rv		200.0 µM	18.0 µM	0.9	**39.6**
Probe		200.0 µM	8.0 µM	0.4	**17.6**
			Total Mix volume:	8.0 µl	352.0 µl
			Sample volume:	2.0 µl	
			Total Reaction volume:	10.0 µl	

Mastermix 2: **NOV**

Required No. of reactions: **41**

Extra mastermix volume: **5.0%**

44 reactions

Reagent	Reagent ID	Initial conc.	Final conc. / reaction	Number of reactions	
				1	44
water		-	-	0.8	**35.2**
Master Mix		2.0 x	1.0 x	5.0	**220.0**
Primer Fw		200.0 µM	18.0 µM	0.9	**39.6**
Primer Rv		200.0 µM	18.0 µM	0.9	**39.6**
Probe		200.0 µM	8.0 µM	0.4	**17.6**
			Total Mix volume:	8.0 µl	352.0 µl
			Sample volume:	2.0 µl	
			Total Reaction volume:	10.0 µl	

Figure 8.7 - Example of automatically generated master-mix calculations report.

developed for guiding users through the assay validation steps or for providing advanced “validation modules” for automated liquid handlers.

Currently, the only MIQE step directly related to routine qPCR runs that is addressed by automation is “qPCR protocol”. The most advanced automated systems available store detailed qPCR protocol information, including the volumes and concentrations of individual reaction mixture components (template DNA or cDNA, primers and probes, DNA polymerase, Mg2+, buffer). Such systems can also calculate the volumes required based on the number of reactions that will be performed, which may change from one qPCR run to the next (see Figure 8.9).

Currently, users must manually set up the liquid handling tasks for each run, especially in research environments where plate setups change from run to run. In such environments, conventional liquid handling automation can easily become cumbersome, as plate setup is time consuming, tedious and a source of error. In the future, automated solutions will probably allow the user to select from lists of samples, qPCR protocol(s),labware, and plate setups, and will include autocalculations for reaction mixtures and dilutions, based on these selections. To allow this, qPCR protocols (information about qPCR assays, qPCR controls, number of sample replicates, dilutions, etc.) will need to be completely separated from run execution.

qPCR experiments are frequently integrated into larger experiments. It is therefore critically important that automated solutions handle information input and output so that the flow of information is not interrupted and that repetitive manual data entry, a frequent source of error, is no longer required. For example, information input can be as simple as simple lists of

samples that can be transferred to a LIMS. Information output can be settings for specific qPCR thermocyclers, including the plate layout and detector information. Such solutions can ensure the following:

- As many steps as needed can be automated, reducing inter-operator variability.
- Information is entered only once and is automatically distributed to databases, downstream files, software, and instruments, reducing the sources of human error.
- Detailed information is automatically collected and stored more quickly and accurately than can be expected from information entered manually, improving traceability.

One such advanced solution that is currently available is the PIPETMAX qPCR Assistant (Gene Expression Signature in Peripheral Blood as a Marker of Parkinson ’s Disease). This qPCR workflow software package ensures reproducible, flexible and traceable qPCR runs. Singleplex and multiplex qPCR runs are easily created based on operator-supplied answers to simple questions about their qPCR experiments. Master mix preparation, sample dilution, and reaction plate preparation are automated with extensive documentation including a qPCR thermocycler set-up file that ensures off-liquid-handler traceability. This package ensures MIQE “qPCR protocol” compliance (see this video).

For researchers that do not have automated liquid handlers, information management systems that facilitate qPCR experiments and support manual pipetting to a high degree are available as well (see Figure 8.5).

Data analysis

qPCR data analysis can be divided into at least two sectors:

- Quality control performed in the qPCR thermocycler software (e.g. baseline settings, threshold settings, amplification curve inspection, melt curve analysis, etc.)
- Quality control performed at the Cq level followed by data analysis that may be performed in independent downstream software

Control	Abbreviation	Purpose of use
Non-template control	NTC	• Identifies possible contamination introduced during setup of qPCR reactions
Negative control of isolation	NCI	• Identifies possible contamination during isolation of nucleic acids from samples
No RT control (negative RT control)	NRT	• Indicates contamination of RNA with gDNA (e.g. in gene expression experiments) • Performed on RNA (not cDNA)
Positive PCR control	PC	• Used to verify correct execution of PCR reaction • A sample that contains (a known amount of) the target sequence is used • Useful for monitoring of assay variation over time
Positive control of isolation	PCI	• Confirms successful isolation of nucleic acids from samples • A sample that contains target nucleic acids is used for PCI
Internal positive control	IPC	• IPC is a rreference qPCR assay targeting NA of host organism (e.g., human 18S rDNA if samples are of human origin host genes) or an artificial target; it is used to make sure that the nucleic acid isolation was successful and to check the inhibition of PCR reaction
Environmental control	OE	• Indicates contamination from environment during isolation
Control of inhibition	CI	• Identifies the presence of inhibitors of PCR reaction • Is used to check the efficiency of amplification

Figure 8.10 - The most commonly used controls in qPCR

Several experimental, environmental, and instrumental controls are required for every qPCR assay and must be reviewed before data analysis can be performed to ensure the sample data are reliable. The most commonly used controls in qPCR are presented in Figure 8.10.

The most commonly automated data analyses are the QC check (review of all controls) and the qualitative check, which determine the presence or absence of a target in a sample. These two steps are sufficient for qualitative diagnostics, and fully automating such systems is relatively straightforward. Some thermocycler manufacturers have included advanced qualitative data analysis modules in n the qPCR thermocycler software. Other laboratories develop their own in-house solutions, which are subsequently incorporated into their LIMS systems or rely on qPCR-specific systems providing full traceability and compatibility with various standards of quality assurance (e.g., GENEIO from BioSistemika).

In addition to the qualitative approach, a vast array of applications is available for quantitative qPCR data analysis, making it possibly the most variable step in the entire qPCR workflow. As a consequence, this step is the least standardized, and fewer automated solutions are available than for the other steps. The data analysis workflow usually starts with initial QC check provided by the qPCR thermocycler software. The Cq data are then exported, re-formatted (if required) and imported into downstream software. In addition to Microsoft Excel and Open Office Calc, qPCR-specific software tools, such as GenEx (Tataa Biocenter), qBase+ (Biogazelle), RealTime StatMiner® (Integromics) and qPCR thermocycler software providing modules for qualitative and quantitative analysis, are commercially

available for this purpose (Pabinger et al., 2014; list of relevant qPCR-related software at gene-quantification.info).

The drawback of combining several different software for data analysis is unavoidable manual manipulation of data, such as sample information entry into qPCR thermocycler software, which is often not performed by users, and importing raw Cq data into the downstream software. When sample names are not initially entered into qPCR thermocyclers, the data must be entered post-run, creating a possible source of error. In some cases, data exported from cyclers must be reformatted or reorganized (e.g., to combine technical replicates, arrange by serial dilutions and group samples from a particular assay). The necessary copying and pasting of information presents an additional source of errors.

To date, no automated system that complies fully with the MIQE "Data analysis" step for all current qPCR applications is yet available.

Other data relevant to qPCR experiments

In addition to the data described above, MIQE guidelines require that several types of information are available for each qPCR experiment, including data from nucleic acid extractions (purity, RNA integrity, etc.), information related to qPCR targets (database accession number of each target and reference gene, locations of primers and probes, sequences and concentrations of each oligonucleotide, BLAST results, etc.), and information about users contributing to the experiment.

Currently, this information is usually written in laboratory notebooks and presents the problems discussed above. An automated qPCR system that enables attachment of external files, such output from instruments performing RNA integrity, will increase traceability and save time for experiments and must be MIQE compliant for publication.

Section 6

Pitfalls of using software automation

The approaches described above improve the ease of performing qPCR, increase the reliability of the results and improve traceability. However, implementing such software into an existing laboratory operation requires investment: both with respect to finances, as the software must be purchased or developed, and time, as implementation can take more than one year. The extent of these investments is largely determined by the software solution chosen.

Implementing automation solutions is costly because every laboratory has their own established system, which likely has several unique aspects, including differences in protocols and reagents, laboratory equipment, regulations, standards as well as to “soft factors”, such as the culture within the institution. Ultimately, each user has his or her own “style”. Off-the-shelf solutions are not designed to meet individual laboratory needs and setup procedures. Therefore, laboratory software providers often customize their system based on the requirements of the laboratory as communicated to the software developers. Such customized solutions are often “buggy”, expensive, non-intuitive and not user-friendly.

Whereas larger institutions can afford the time and effort to implement customized systems, small laboratories or research groups, which may have only a dozen members, cannot afford to halt experiments for an entire year and do not have the

budget for the laboratory software. To address the needs of such laboratories, a new kind of software must be developed that is easy to implement, provides basic features and allows intuitive customization. Such solutions should cost significantly less than solutions for large laboratories and should be successfully implemented in matter of days with the necessary early customer support.

An often overlooked yet important aspect of new software implementation is the acceptance of the software by the operators and other users. This is especially important when the cost of the software requires a decision at the management level. Operators that feel the new software inhibits their work routine, is not intuitive, or does not contain features they need, often bypass the software or use the old system, manually entering the data into the software. Lack of acceptance can undermine the usefulness of the software and add other costs to the purchase price.

The complexity of the software can also introduce important issues. Some systems require extensive training for the people who operate them. In laboratories with high employee turnover, knowledge transfer about operating these systems can be problematic. Instruments with simple and easy-to-use software provide obvious advantages. The ideal automation software should be easy to integrate into existing systems, easy to use and offer effective return on investment (ROI). Such challenges can be observed for example in the existing ELN market (Machina et al., 2013a).

Figure 8.11 - Simplified qPCR automation through application based software

Section 7

Arguments for investing in advanced automation solutions ensuring traceability and reducing inter operator variability

As discussed above, advanced automation solutions bring several advantages to qPCR laboratories, including improved traceability, increased comparability between runs, and improved reproducibility of results. However, despite these advantages, the return on investment (ROI) must be considered by every laboratory before purchase.

The cost of failures that threaten compliance to regulations in industrial and regulated environments is usually very tangible and extremely high. Therefore, the investment for advanced automation solutions is easily justified in these environments.

In contrast, costs of errors or failures are relatively low in scientific research laboratories and the ROI is more difficult to estimate. However, it is becoming increasingly expensive to perform qPCR and newer techniques, and granting agencies are increasingly setting requirements for standardization (Collins et al., 2014; see also the European Pharmaceutical Review qPCR in-depth focus). In this new environment, scientists are starting to view traceability and standardization as improvements rather than obstacles to scientific freedom, and, correspondingly, advanced automation solutions are becoming more common in these laboratories that strive to achieve the highest levels of verifiable science.

Section 8

Conclusions

qPCR plays an important role in various areas of basic and applied science such as drug discovery, advanced diagnostic methods, systems biology, etc. which are frequently funnelled to industrial and service settings. Results generated by users therefore need to be trustworthy and verifiable. This is increasingly being adopted and requested by major grant-awarders including the NIH. How is verifiable data generated? Through thoughtful experimental design, by careful and traceable execution of lab experiments performed with quality instruments, and the use of integrated and sophisticated software programs. The most fundamental approach in achieving verifiable science in the laboratory is standardization of experimental procedures. In the qPCR arena this was achieved by introducing the MIQE guidelines. In this increasingly digital era, traceability of not only the lab workflow, but of the immense “big data” generated and analysed is becoming increasingly important. By implementing traceability we can identify errors more rapidly and take measures to avoid them in the future.

We are entering into an age where scientists will be required to use more sophisticated experimental techniques, but will have less time to generate reliable results. Therefore, the scientific community will need equipment and tools that more easily enable verifiable science. . This points to the development of automated and integrated workflow solutions that incorporate standardized experimental procedures as well as validated data acquisition and management software plans.

Section 9

Literature

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Application Notes

Precise Evaluation of Plant RNA Extraction Methods with Automated RT-qPCR Assay Preparations (Application Note)

Normalization of DNA and RNA Samples with PIPETMAX® Normalization Assistant (Application Note)

Expression of Oxidative Stress Genes in Human Stem Cells in Physiological Versus Atmospheric Oxygen Levels (Poster)

Gene Expression Plant – Virus Study Monitoring Potato Virus Y (PVY) with Real-Time PCR (qPCR) Results Using the PIPETMAX® 268 (Application Note)

Gene Expression Signature in Peripheral Blood as a Marker of Parkinson's Disease (Application Note)

Chapter 15

MIQE Guidelines and microRNA

How to overcome the challenges of qPCR on short RNA targets and generating accurate and reliable data

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Section 1

Summary

MicroRNA qPCR is a very powerful and accurate tool for quantification and profiling. In many ways, microRNA profiling is similar to any other kind of RNA profiling using qPCR. In other ways, however, the experiment needs to be adapted to fit the unique challenges associated with microRNAs.

In this chapter, we will present some of these challenges and how they can be resolved. Learn how to set up and perform your microRNA qPCR experiment according to the MIQE guidelines. Find out what controls to choose and how to avoid technical and biological variation. Get valuable tips on how to work with complex samples, such as FFPE and biofluids, and how to perform the normalization and data analysis.

By following the tips and guidelines presented in this chapter, you will be well-equipped to setup your own MIQE-compliant microRNA qPCR experiments to get the most out of your samples.

Section 2

Introduction to microRNA

microRNAs are very short regulatory RNA – typically around 19-23 base-pairs long. They are of interest to researchers for various reasons: for one, they have turned out to be important in gene regulation, but just as importantly their expression levels may change with disease states making them excellent biomarkers, e.g. in cancer (Lu et al, 2005). They are more stable compared to other RNA species, mainly because of being part of protecting protein complexes. Furthermore, microRNA are secreted to the bloodstream where they are found in protein complexes, exosomes – protecting them from the otherwise rapid degradation of RNA in blood (Cortez et al, 2011; Arroyo et al, 2011; Valadi et al, 2007).

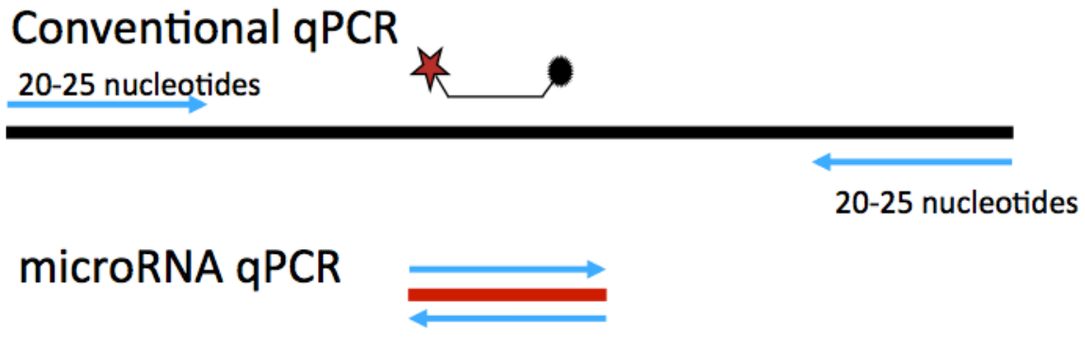
microRNA are excellent biomarkers

Learn more about microRNA as biomarkers and microRNA in biofluids including serum/plasma.

Read how other research scientists work on developing microRNA disease biomarkers.

As interesting as microRNAs are, they are also difficult to detect. The first hurdle is their small size. In qPCR, one would typically design two primers each of roughly 20 nt which is the same length as a microRNA. If using probe-based detection, the probe should be localized in a third, independent location for added specificity. For obvious reasons, this is not possible with such short targets (Figure 15.1 - A). Adding to this, many microRNAs are closely related in families where sequences only differ by one or two nucleotides, making it difficult to design discriminatory assays (exemplified by the let-7 family shown in Figure 15.1 - B). Finally, the GC content varies greatly between different microRNAs (Figure 15.1 - C).

Figure 15.1 - microRNA are particularly difficult to detect using qPCR for several reasons

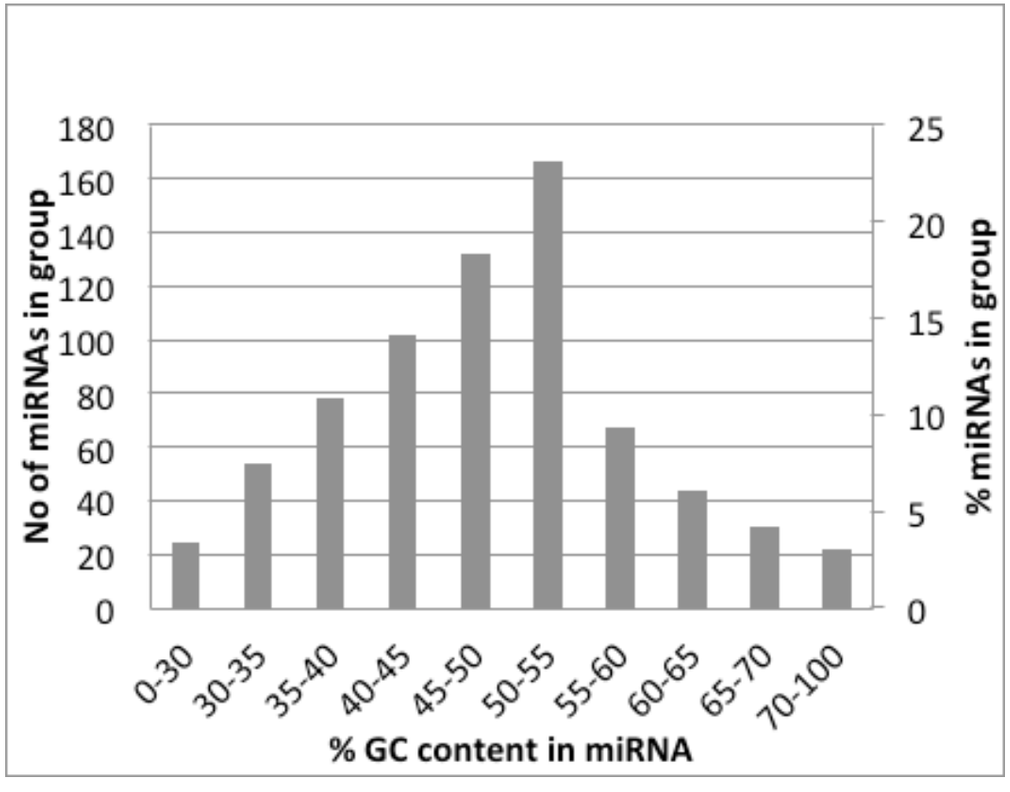


A - Due to their short length, conventional qPCR design strategies don't work. There is no room for two full-length primers, let alone a separately located probe.

qPCR detection of microRNA is more difficult than for longer RNA

There are examples of microRNAs with GC content over 90% (Figure 15.2), in which case it is difficult to design assays without too high self-complementarity or off-target cross-reactivity. In the other end, some microRNAs have as little as 25% GC. In these cases, using the entire microRNA sequence for one primer results in a Tm as low as 50ºC, making it unsuitable for qPCR under normal amplification conditions (see online presentation for details).

Figure 15.2 - Overall GC distribution amongst all microRNA sequence



Nevertheless, several strategies have been employed to overcome these general challenges in microRNA detection. Over the past few years qPCR has become the most widely used method for the study of microRNAs. It is fast, extremely sensitive and offers linear detection over several orders of magnitude. microRNA qPCR arrays can profile microRNAs on panels in a matter of hours and offer linear ranges of up to 7 orders of magnitude (example: miRCURY LNA™ Universal RT microRNA PCR). The most sensitive systems allow individual microRNAs to be accurately quantified from as little as 1pg total RNA. This level of sensitivity enables microRNA profiling from difficult samples such as FFPE, samples collected using laser capture microscopy, and biofluids including serum, plasma and urine. However, in order to get biologically relevant results, it is important to set up the qPCR experiment correctly.

LNA™ Technology for microRNA qPCR

Learn how the LNA™ Technology can improve the sensitivity and specificity of microRNA qPCR primers:

Watch the LNA™ movie

Improving performance of microRNA qPCR. Four good reason to use LNA™-enhanced qPCR primers

When studying microRNA expression in a biological setting, e.g. when comparing different disease stages, even very small changes in microRNA expression levels may be biologically significant. Yet, it will require a sufficient number of samples and correct normalization to reveal the differences with statistical significance. An insufficient number of replicates may obscure discovery of small but important differences. Poor normalization can lead to incorrect conclusions regarding the magnitude of regulation and even direction of fold change when studying differential expression. Proper study design and reliable normalization is therefore critical when analyzing differences in microRNA expression.

"Even very small changes in microRNA expression levels may be biologically significant"

The MIQE guidelines (Bustin et al, 2009) serve as a check-list on which parameters to consider and document when performing qPCR experiments. In this chapter, we will go through the steps involved in setting up a microRNA qPCR study in compliance with the MIQE guidelines and explain how to perform the normalization and data analysis.

Much of what is described in this chapter is also presented in this webinar.

Section 3

Setting up the study

1. **Choosing your format – screening versus validation**
2. **The optimal sample size of a qPCR study**
3. **Determining the number of replicates in each step of the study**
4. **Selection and use of controls for optimal data validation**
5. **Quality control using SYBR® Green**

There are several important parameters to consider when setting up a qPCR study to ensure obtaining correct data. In this section some of the most important parameters will be discussed, such as array format, sample size versus sampling method, number of replicates and types of controls.

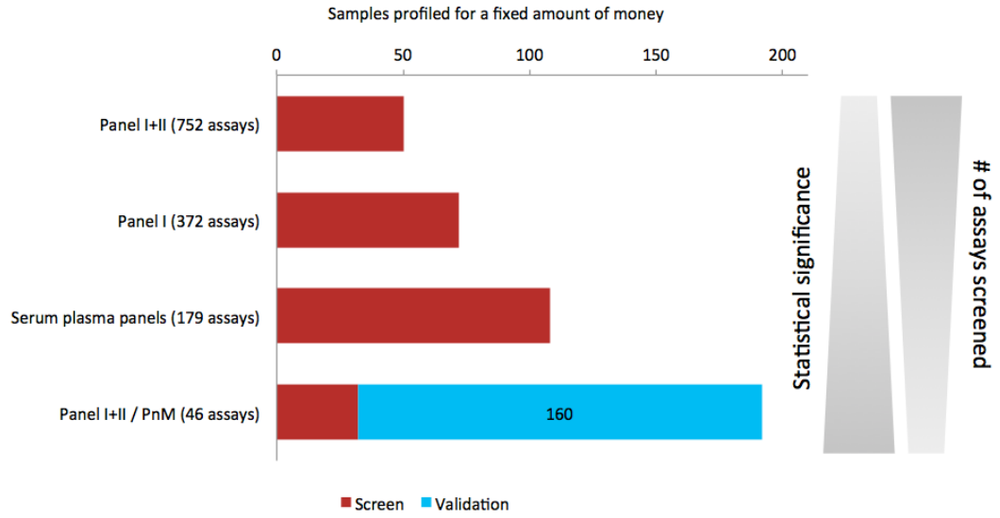
Note that the revised MIQE guidelines state that you should provide, as a minimum, the sequence of each qPCR amplicon when publishing. For microRNA analysis, the sequence of the microRNA targeted is considered sufficient. However, do note that miRBase updates may involve changing the microRNA sequence and/or name while keeping the accession number constant. For this reason, it is important not to provide the microRNA name or miRBase accession number only, but the actual microRNA sequence the assay was designed for.

Try miRSearch to learn more about your microRNA of interest. Find historic miRBase annotations for any microRNA.

Choosing your format – screening versus validation

In setting up, first of all you need to decide on the format of your microRNA qPCR study. If you are screening samples without any pre-existing knowledge of what results to expect, then a large number of targets is desirable (e.g. a full miRNome panel). The purpose of the screening is to select targets of relevance for subsequent validation. However, screening a large enough set of samples for strong statistical answers on a large number of targets (>700) may be prohibitively expensive. To overcome this hurdle, you may apply the strategy of initially screening a relatively small sample size, in acceptance of weaker statistics. The strong statistics should then be obtained with a reduced number of selected targets on a panel of custom selected microRNA assays, but using a much larger sample size (Figure 15.3).

Figure 15.3 - Strategy for combining full miRNome screening with sufficient sample size. By doing an initial screen on full miRNome panels and selecting a subset of assays for validation, a much larger sample size can be profiled for the same amount of money, thereby increasing confidence in your results



The optimal sample size of a qPCR study

What is the ideal study sample size? The answer to this question is not simple. The sample size needed to get a statistically relevant answer from the study depends both on the inter-sample variance, the introduced technical variation, and on the expected amplitude of differences the study is intended to show. If looking for very large differences in a homogeneous sample type where a low biological variance can be expected (i.e. 10-fold changes in treated versus non-treated cell culture), you may not need a large data set to show differences that are both biologically relevant and statistically significant. However, when searching for small differences in a very heterogeneous sample set where a high biological variance can be expected (i.e. two-fold changes between disease states, measured on biopsies from a tissue type with a complex cell content from a diverse set of human individuals) you may need a very large number of samples within each group in order to obtain statistical significance for the biologically relevant differences.

If you have knowledge of the expected biological and technical variation in the study, performing a power analysis can be a very helpful tool to evaluate the number of samples needed. The power analysis can be performed in a simple way e.g. using the experimental design feature in a data analysis software such as GenEx qPCR analysis software, or one of many free online tools (an overview of tools can be found at statpages.org).

Determining the number of replicates in each step of the study

The purpose of performing replicates of any test is to remove noise caused by biological variation and technical variation, and calculate statistical confidence intervals. It is important to evaluate the necessity of including both biological replicates (e.g. individuals and/or samples) and technical replicates (e.g. RNA extractions, RT and PCR reactions) in the study.

"The number of replicates should never be lower than three"

Which replicas should be performed depends on which steps introduce the most variation. The number of replicas depends on the level of variation, but should never be lower than three. If only two replicates are performed, and they vary widely, it will be impossible to determine which one is the outlier – three replicates

allow identification of outliers, and allows performance of statistical tests on the data.

Minimizing biological variation

Biological variation arises mainly from the differences between individuals. The aim of a study is usually to identify differences between two or more biological groups. Biological variation within groups may be low if the group to be studied is very homogeneous (genetically and environmentally). This could be true for in-bred animal strains, and if the specimen of interest is cell culture the variation may be close to negligible. On the other hand, if the population to be studied is very heterogeneous, the biological variation within groups could be very large and therefore necessitates a high number of biological replicates. Heterogeneous populations are samples collected from a general population which could include a mix of ethnicities. Hence, samples from clinical studies including microRNA biomarker development studies are typically heterogeneous.

In summary, designing a study on a very homogeneous biological population has the advantage of keeping biological variation within groups low, thereby allowing the identification of small differences even within a reasonably low number of biological replicates. This, of course, is at the risk of hiding important biological differences in a larger population. On the other hand, using a heterogeneous study population will increase the chance of discovering important differences within the groups – but the higher level of variation may result in the need for a prohibitively large number of biological replicates in order to reveal these differences.

Figure 15.4 - Main sources of technical and biological variance

Sampling	Low-high, depending on biological material*
Sample preparation	Medium due to technical variation/ efficiency etc.
RT Reaction	Low, from pipetting, lot to lot correlation
PCR RT Reaction	Low, from pipetting, lot to lot correlation

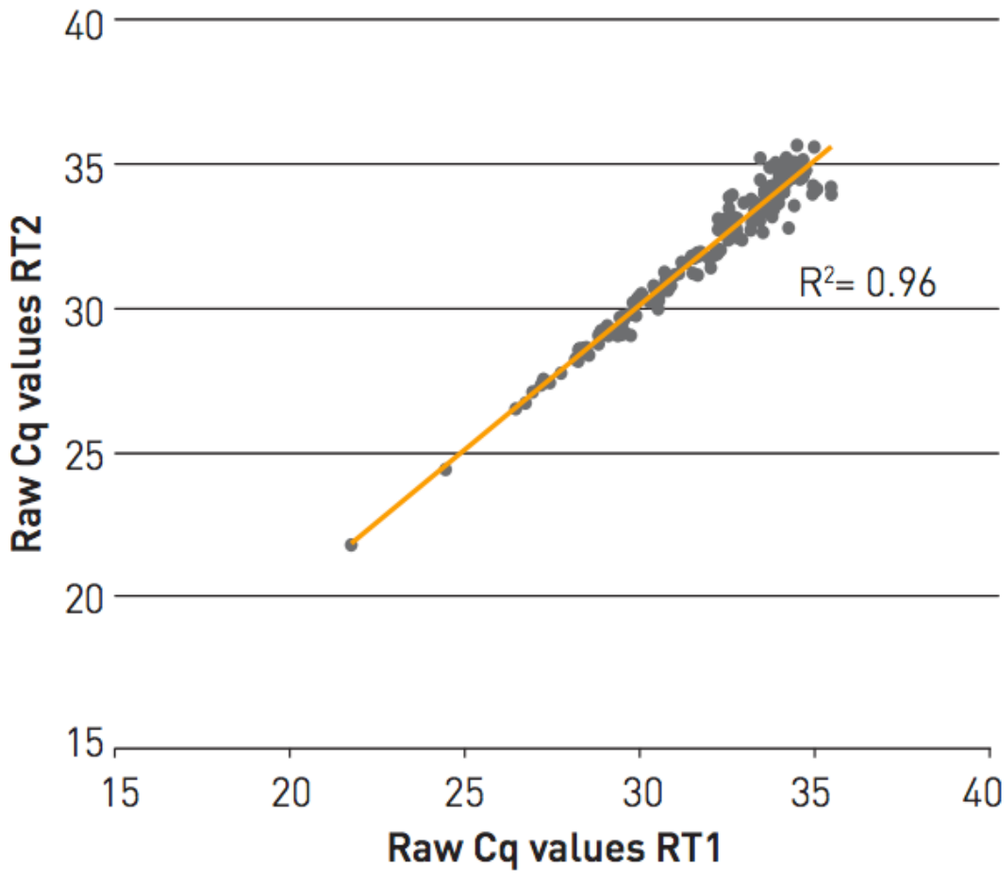
*Technical variance - *complex samples containing mixed cells as vascular and epithelial cells introduce high variance*

Avoiding technical variation

No technical manipulation of samples is performed exactly identically each time. The result of this is introduction of a certain level of variation with each step of sample manipulation. This can be countered by performing technical replicates. In general, qPCR studies comprise many different experimental steps and thus many potential points for introduction of technical variance. This is also the case in microRNA qPCR (Figure 15.5). Which types of technical replicates make the most sense to perform depends on the relative levels of variation each step introduces (Figure 15.4). For example, if the extraction procedure introduces a lot of technical variation while the PCR step is very reproducible, performing replicates at the PCR level may not improve the dataset – while replicates at the extraction level may remove a significant amount of noise and thereby reveal statistical significance to biological differences.

"A robust qPCR platform will help minimize technical variation"

Figure 15.5 - Excellent reproducibility between RT reactions on total RNA from serum. Raw Cq values from two separate RT reactions (RT1 and RT2) on total RNA purified from 65 µL serum are shown. A total of 730 microRNAs were profiled. Only microRNAs with Cq values below 35 have been included (133 data points).



Sampling is a technical step which is often ignored, but mentioned in the MIQE guidelines for a very good reason. The process of sampling and subsequent handling prior to extraction may in fact introduce significant technical variation often confused as biological variation. If the sample to be studied is a biopsy taken from a complex tissue type with many different structures and cell types (such as the kidney), three samples taken from the same individual may well be very different. On the other hand, three consecutive blood samplings from the same individual, taken at the same time, would probably be quite

similar unless hemolysis was introduced at phlebotomy. Similarly, the speed and method of sample conservation as well as the length of storage may highly influence the data obtained (find tips and information for sampling biofluid samples here)

Another point to consider in the design of a study is the RNA extraction method used. Different extraction methods may introduce different levels of variation, thus affecting the need for replicates. This becomes particularly important if you are interested in microRNA expression in biofluids such as serum, plasma, urine or cerebrospinal fluid (McAlexander et al, 2013). In most sample types, the majority of RNA comes from the larger RNA types such as mRNA, tRNA and particularly rRNA. However, if the sample is a virtually cell free biofluid, no large RNA species are present, whereas microRNAs are sufficiently expressed for an analysis to be feasible. Guidelines for profiling of microRNA in serum/plasma and other biofluids are found here.

As recovery of RNA is never 100%, starting out with a low level of total RNA of which the microRNA composes a large proportion can result in a significant loss of microRNA during extraction, resulting in lower yield and higher variation in your experiments. This may be improved by adding a microRNA-free RNA carrier, e.g. bacteriophage MS2 total RNA or yeast tRNA (Andreasen et al, 2010).

Carrier RNA

The purpose of adding carrier RNA during the extraction is to increase the total RNA amount as well as the RNA complexity in a sample as detailed below.

The increased RNA concentration will have the effect of minimizing loss of specific RNA template due to stickiness of plastic, extraction filters etc. This is particularly useful in samples types with low concentrations of total RNA such as serum/plasma or synthetic RNA targets.

The increased complexity works to increase low-energy DNA duplex formation, thus competing for undesired unspecific bindings such as primer-dimer formation. Examples of carrier RNA not containing microRNA are MS2 bacteriophage total RNA and yeast tRNA.

"Minimize microRNA loss by adding carrier RNA"

Also to consider is the quality of the sample RNA. The MIQE guidelines require measurement of RNA integrity. For microRNA, there are several things to consider when looking at RNA integrity. The first is that measuring the RIN or similar will not tell you

whether or not microRNA was degraded, since 1) they are already in the short end and 2) degraded longer RNA will show up around the size section containing microRNA.

Furthermore, microRNA are known to be more stable, so degradation of longer RNA may not necessarily mean that the microRNA fraction is compromised too. However, degradation of microRNA may still take place, and measuring integrity will make you aware if this is something that should be considered in the data interpretation – but just as importantly highly degraded RNA increases the risk of unspecific amplification for some detection platforms (Becker et al, 2010).

Apart from measuring RNA integrity, a good way of estimating the quality of sample is by analyzing a small set of selected endogenous and spike-in control assays prior to larger screening (see **Figure 15.7**, and the miRCURY™ microRNA QC PCR Panel instruction manual). This will be indicative of issues with the sample preparation and/or qPCR inhibition, and will allow omitting samples of compromised quality from a large, costly screening, and thereby allow saving resources.

RNA spike-ins

It is important for any qPCR experiment to ensure that the quality of the input RNA is sufficiently high for effective amplification. By introducing spike-in RNAs during RNA isolation and cDNA synthesis and comparing the qPCR results to those from endogenous reference genes, important information regarding the quality of the samples can be obtained

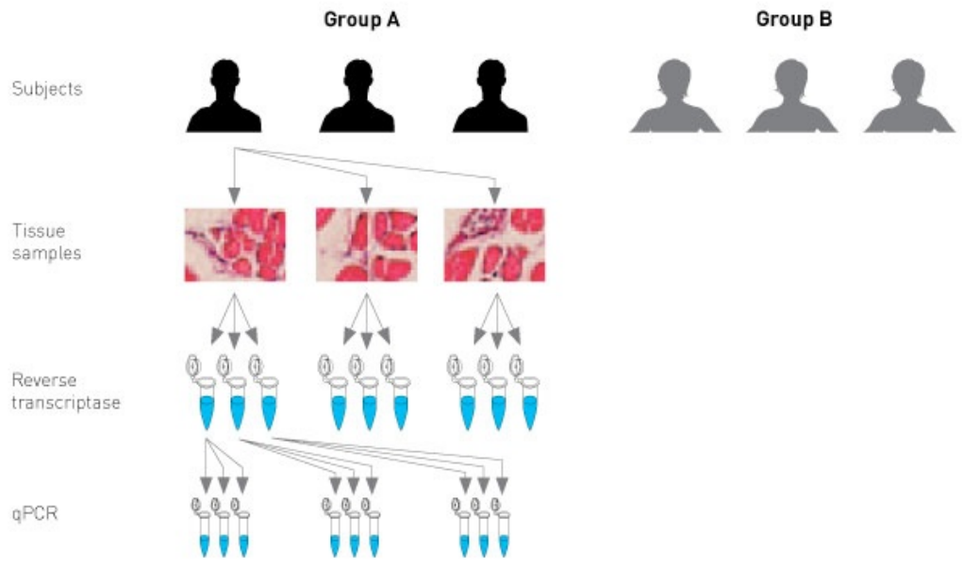
Learn more here.

Determining the amounts and sources of variation: Nested ANOVA

Setting up a large experiment may be both costly and time consuming. If you already know the sources and magnitudes of variation in your study, the previously mentioned power analysis (0) can help you calculating the number of samples and replicates needed. However, if you do not have this type of knowledge it may be helpful to perform a smaller pilot study with the purpose of determining these factors. From this it can be decided which replicates to perform, and how many biological samples are needed within each group. Such a pilot study can be performed through a nested design where three replicates are performed at each potential level of variation and the results are analysed using a nested ANOVA (Tichopad et al, 2009, see **Figure 15.6**). If

you are using GenEx for your data analysis, you can also find help in designing and analysing your nested experiment here. Furthermore, good discussions and help can be found at www.qPCRforum.com.

Figure 15.6 - Example of an experimental setup. Proposed pilot study to determine the contribution to variation at each technical or biological level. This nested study uses triplicates at each level and can be analyzed using a nested ANOVA.



Cq values

Different types of qPCR cyclers use different terms for their metric of quantification (Ct, Cp, TOP). In line with the MIQE guidelines suggestion, the standardized nomenclature quantification cycle (Cq) covering all of those therms are used here.

Selection and use of controls for optimal data validation

In order to ensure that experiments have performed well, and enable troubleshooting if they have not, it is always a good idea to include a number of controls. How many and which are relevant depends on the type of study and the main risks inherent to the study. The experimental controls include various types of negative and positive controls.

Setting up the correct negative controls

Negative controls are important for two purposes of equal importance: One is to reveal potential contamination in the experiment, and the other is to determine background levels for each assay. Background may arise from contamination, primer-dimer formation, or non- specific amplification and can result in an amplification curve with a high Cq in a SYBR® Green assay. The different types of negative controls reveal different potential issues.

Overview of negative controls explained in the following:

→ No template control (NTC)

→ No enzyme control (NEC)

→ Mock RT reaction

The no template control (NTC)

A No Template Control (NTC) in the PCR reaction (using water or buffer as template) will reveal if the water or master mix used has been contaminated. An NTC in the Reverse Transcription (RT) reaction (RT on water or buffer) will equally reveal if the RT reagents have been contaminated (but it will not be possible to discriminate between contamination of RT reagents or PCR reagents). Both types of NTC will also reveal if primer-dimer is an issue. A blank purification may be used for studies involving biofluids. It should be noted that because the primers are present in relative high concentration, they may show increased tendency to form primer-dimers relative to when a template (RNA and DNA) is present. This type of negative control can thus be too stringent.

The no enzyme control (NEC)

This is an RT reaction performed without enzyme, but with RNA template. It will reveal if there is any DNA contamination in the RNA sample (either genomic or amplicon from previous experiments). DNA contamination is not considered a large problem with microRNA analysis, since virtually all the available methods are essentially free of detecting genomic DNA. At the same time, the RNA in the NEC will act as carrier and help prevent primer-dimer formation. Thus, the level of primer-dimer (if any) is likely to better resemble the level of primer dimer formation in a biological sample than is the case for the NTC.

The mock RT reaction

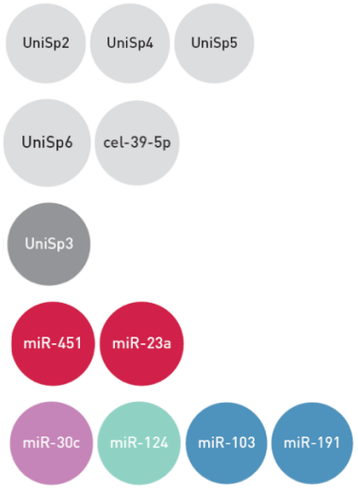
This is an RT reaction carried out on carrier RNA only. It may reveal unspecific amplification levels. If performing this control, it is important that the carrier RNA is free of any microRNA. Bacteriophage MS2 total RNA or yeast tRNA are examples of commonly used carrier RNA. This control becomes particularly important in experiments where carrier RNA has been used in the extraction, as it will reveal if the carrier used causes increased background. An even better option for the mock RT control may be to perform a mock extraction (extraction of carrier only), and use this as template in an RT reaction, corresponding to an NTC. This will additionally reveal any potential contamination during extraction.

Purpose and use of positive controls

The purpose of positive controls is to allow monitoring the quality of the extractions, samples and reactions, and to aid in troubleshooting if the results look sub-optimal. There are several options for good positive controls. Figure 4 shows an example of how positive controls can be employed as quality controls, using a small selection of endogenous and spike-in controls.

Figure 15.7 - Quality control of samples by PCR (RNA PCR QC). miRCURY LNA™ Universal RT microRNA QC PCR Panels are available as ready-to-use PCR plates (96-well or 384 well).

- Monitor RNA isolation efficiency (add RNA spike-ins to lysis buffer)
- Monitor RT and PCR inhibitors (add RNA spike-ins to cDNA synthesis)
- qPCR monitoring (inter-plate calibrator, IPC)
- Unique hemolysis indicator ΔCq(miR-23a – miR-451)
- Biologically relevant endogenous microRNAs



Overview of positive controls explained in the following:

→ Synthetic spike-in RNA in RT reaction

→ Synthetic spike-in RNA in the extraction

→ microRNA specific positive controls

Synthetic spike-in RNA in RT reaction

Adding a synthetic RNA template as spike-in in the RT reaction will allow checking if the cDNA synthesis has worked well. In addition, RNA spike-ins can give indications for the presence of inhibitors, either in the RT or PCR reaction. Not all assays are equally sensitive to different inhibitors. This means the presence of some inhibitors may not be revealed by the spike-ins. Therefore, it would also be recommend monitoring the efficiency of all assays, as described in the subsequent section “Quality control using SYBR® Green".

Synthetic spike-in RNA in the extraction

A spike-in RNA template can also be used to monitor the uniformity and efficiency of the extraction procedure. In this case, the spike-in should be added to the sample lysate with or immediately after the lysis buffer – otherwise, degradation of the spike-in is a significant risk. The spike-in(s) added to the extraction should be different from those added to the RT reaction – otherwise it will not be possible to discriminate

between problems with the extraction and problems with the RT or PCR reaction.

microRNA specific positive controls

In case of no amplification signal in the qPCR, troubleshooting may be carried out to discriminate between a true negative (the microRNA of interest is not present in the sample at a detectable level) and a malfunction of the assay using a sample known to be positive for expression of the given microRNA. In this process, it should be kept in mind that validation may reveal different results from a screening, particularly if different methods were used. If a certain tissue or cell type is known to contain a high level of the microRNA of interest, a good quality commercially available RNA sample may be used as positive control.

However, not all microRNA species are expressed in all cell and tissue types, and it may prove difficult to find a biological sample to act as positive control for certain microRNAs. In this case, a synthetic RNA template can be used. Since microRNA are so short, and the target sequence for each assay should always be provided by the assay supplier, it is a simple matter to order selected synthetically generated microRNA from any major oligo house.

Quality control using SYBR® Green

In addition to negative and positive controls, assay properties such as amplicon melting temperature (Tm) and amplification efficiency may be used for quality control.

If you are using a SYBR® Green detection system, you get an option not available with probe-based systems: generating dissociation curves. The dissociation curve gives you two types of information to use in QC:

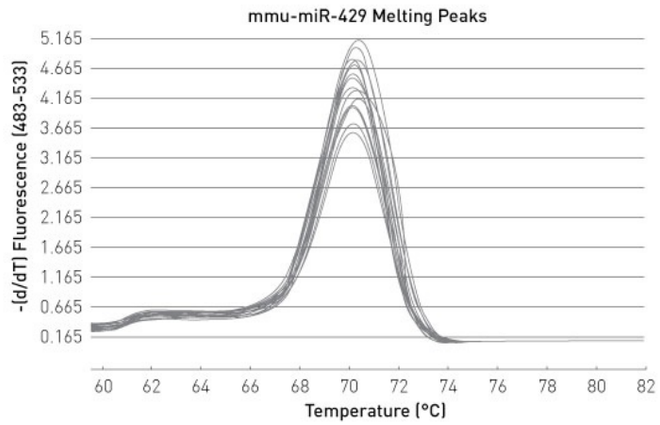
→ One is the shape of the dissociation curve, which should always be a single, clean peak. Keep in mind that the target sequence available for microRNA assay design is very limited, and some extend of potential cross-reactivity in a subset of sample types cannot always be avoided. However, the shape of the melting curve should give you an indication if this is a problem for your assay(s) of interest, in your particular sample(s).

→ The second is the Tm of the specific amplicon which should be reproducible from run to run. Therefore, the Tm can be used to verify that the same amplicon is amplified from sample to sample.

Figure 15.8 shows an example of the melting curves for the mmu-miR-429 assay across 8 different samples. It should be noted that different cyclers may have different temperature calibrations, and this can cause Tm to differ from instrument to instrument. For this reason, the expected Tm of each amplicon should be determined on the instrument used, using a good positive control. In addition, be aware that some instruments may have a slight temperature difference across the heat-block (a rim effect), giving slight Tm variations depending on assay position in the plate. This can be checked by running an entire plate with the same assay, and same sample – or even better, by having the instrument tested for this parameter.

"SYBR® Green based qPCR systems allow readily QC by dissociation curves"

Figure 15.8 - Melting curve analysis is useful for assay quality control. Dissociation curves for one of the miRCURY LNA™ Universal RT microRNA PCR assays across different samples. The shape of the curve and the reproducibility of Tm (peak melting temperature) can be used as quality control. The melting curves show only one major Tm peak corresponding to a well-defined Tm of the amplicon demonstrating specific amplification of mmu-miR-429.



Assay efficiency as quality control

The assay efficiency can be calculated based on the amplification curve using e.g. LinRegPCR or similar algorithm (Ruijter et al, 2013) LinRegPCR can be downloaded from http://www.hartfaalcentrum.nl. A reduced PCR efficiency compared to the expected (as determined using a good quality positive control) may indicate the presence of inhibitors in the sample. Just as for the Tm, the efficiency should be determined on the actual study data.

"Reduced PCR efficiency may indicate presence of inhibitors in the sample"

Some assays may be more sensitive to inhibition than others, and thus it is quite possible to experience inhibition of only a subset of assays when used for microRNA detection in the same sample.

Section 4

Sensitivity of detection system

1. Limit of detection
2. Detection of microRNAs expressed at very low levels

The true limit of detection

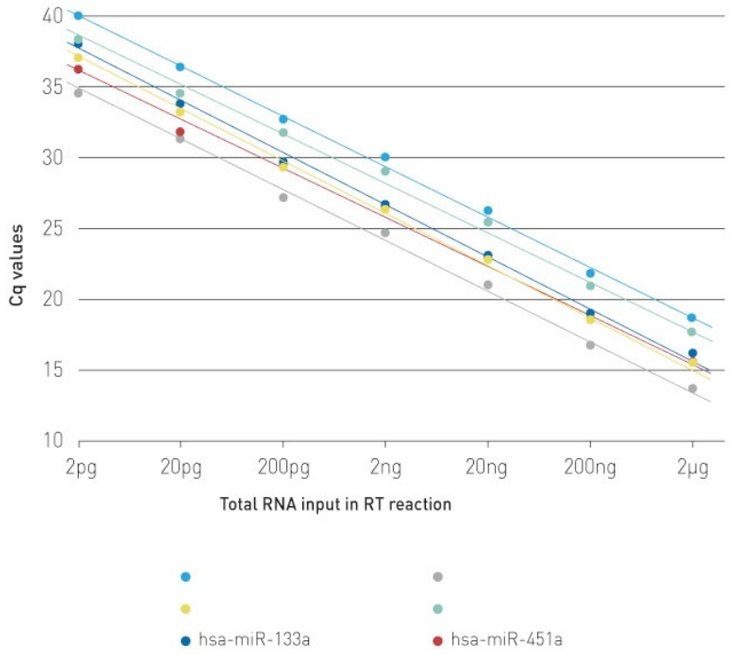
For most researchers involved in microRNA research, assessing the sensitivity of the detection system is not relevant. However if you want to understand the detection system fully, or if you want to generate 100% MIQE compliant data, it is recommend to consider this aspect too. It is a common misconception that lower Cq values from qPCR assays mean higher sensitivity. A Cq value has to be compared either to another Cq value in another sample (leading to a calculation of relative expression) or to a standard curve where known amounts of the target has been analysed (absolute quantification). In order to find out what the true sensitivity of a qPCR assay is, it is necessary to run a dilution series of known input amounts including a negative sample where the template is not present. The limit of detection is usually defined as the last point on that curve where the curve is still linear (e.g. any point that does not lie on the linear regression curve must be excluded).

More than 95% of the primer sets available in Exiqon's miRCURY LNA™ Universal RT microRNA PCR portfolio are sensitive down to the equivalent of 10 RNA copies in the PCR reaction. However, if you wish to determine the limit of detection of a specific assay in the sample types of your experiment, you will need to perform a dilution series (Figure 15.9). This is also the more commonly used method for efficiency calculation (where LinRegPCR is an alternative). A dilution series is a necessity if planning a study with absolute microRNA quantification.

"it is a common misconception that lower Cq values from qPCR assays mean higher sensitivity"

Before performing this experiment, you should decide whether a cDNA dilution series is sufficient, or an RNA dilution series is more appropriate. cDNA dilution is both easier and more cost effective, since you only need to make one RT reaction which is then diluted. When diluting the RNA, you will need to perform multiple RT reactions – but you will also take into account the potential reduction in RT efficiency on dilute RNA samples, the detection limit of the RT step, and potential inhibitors of the RT reaction. All of this information will be lost in a cDNA dilution series – and for some platforms the limiting step for sensitivity is indeed the RT reaction, rather than the subsequent qPCR. This latter can account for observations of low Cq values (strong signal) combined with low call-rates (few positives).

Figure 15.9 - Accurate quantification from 1 pg total RNA starting material. Data from the amplification of 6 microRNAs in serial dilutions of human AM6000 total reference RNA are shown. All microRNA assays exhibit linear read-out with correlation coefficients R(2) > 0,99.

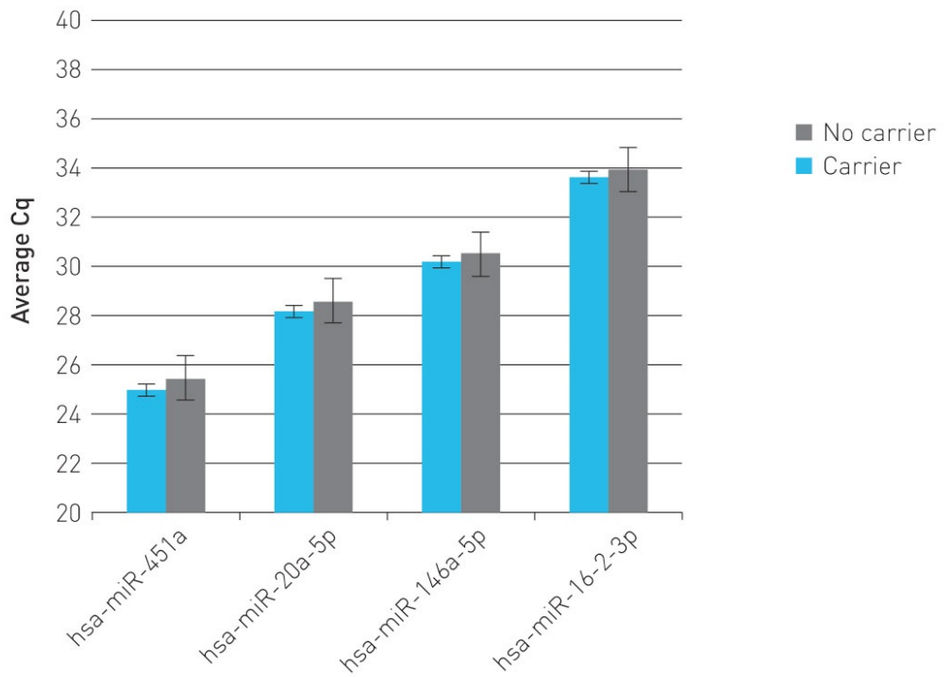


When diluting the RNA sample, we strongly recommend diluting into a carrier RNA (such as bacteriophage MS2 total RNA) to avoid loss of RNA due to plastic adhesion (Figure 15.10).

Detection of microRNAs expressed at very low levels

If the microRNA(s) of interest is expressed at very low levels, the microRNA may fall below the limit of detection in the qPCR reaction. This may be circumvented by increasing the RNA sample input in the RT. However, if there is even a very small amount of PCR inhibitors present in the sample, increasing the RNA input may prove detrimental to the assay performance. In challenging samples such as serum and plasma, characterized by low levels of microRNAs combined with presence of PCR inhibitors, the Cq values obtained will often be in the high end (in the range 30-35). This is perfectly acceptable as long as the signal can easily be discerned from the background. We recommend keeping the amount of RNA input at 20% v/v in an RT to avoid the risk adding inhibitors to the RT and PCR reactions. Increasing the amount of cDNA input in the PCR reaction (i.e. diluting the cDNA less) should be done with caution, as the RT buffer contains chemicals that may inhibit the PCR if present in too high concentrations. Our experience with these types of complex samples is that, for our system, the cDNA should not be diluted less than 40x (compared to the recommended 100x) before addition to the qPCR reaction. For good quality RNA purified from tissues or cells, we have been able to increase sample input by up to 2 µg (compared to the recommended 20 ng) without inhibition.

Figure 15.10 - Adding carrier to the RNA extraction increases reproducibility between isolations and leads to more consistent yields. Carrier RNA improves RNA isolations from serum/plasma.



Section 5

Particular consideration with difficult samples

Certain samples are more difficult to work with when interested in obtaining reliable microRNA quantification and profiling data. Below we will discuss what to be aware of when detecting microRNA in:

→ FFPE samples

→ Serum and plasma samples

→ Urine, CSF and other biofluids

FFPE samples

While FFPE samples fixate protein structure, the nucleic acids are severely degraded. As microRNAs have much better stability in these sample types than the longer RNA species (Li et al, 2007) it is plausible to use these sample types in microRNA studies (**Figure 15.11**). It is important to use an extraction protocol adapted for microRNA extraction.

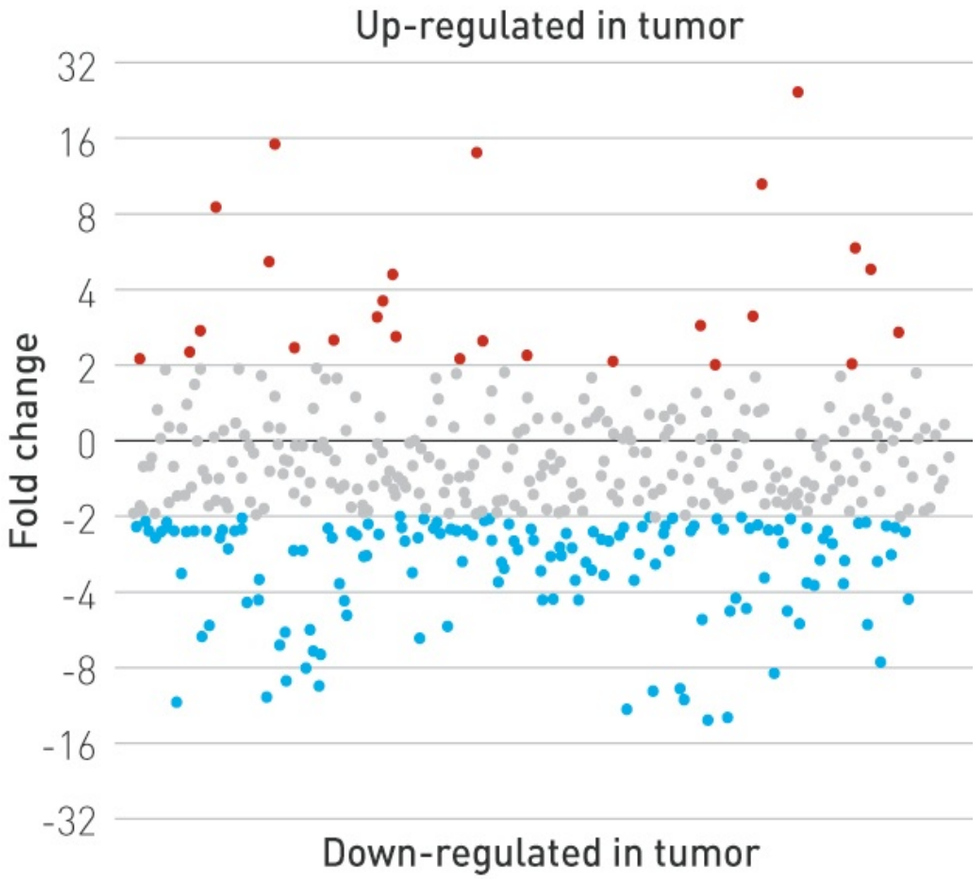


Figure 15.11 - microRNA expression profiling using 40 ng total RNA from tumor and normal FFPE sections using Exiqon's miRCURY LNA™ Universal RT microRNA PCR system, miRNome panel I and II. Data are shown as fold change in expression in tumor compared to normal. Out of 424 microRNAs expressed, 144 were > 2-fold down-regulated in the tumor (blue) and 26 were > 2-fold up-regulated in the tumor (blue).

Serum/plasma samples

As mentioned in the introduction, microRNA has been found to be very stable in the bloodstream, protected by protein complexes or in exosomes, thus allowing measurements of microRNA from serum or plasma. However, there are a number of precautions to take in order to obtain high quality results.

Tips: how to avoid hemolysis

- *Use good and consistent collection device throughput study (e.g. BD Vacutainer)*
- *Follow manufacturer's instructions!*
- *Avoid drawing blood from a hematoma*
- *Avoid frithing the sample*
- *Make sure the venipuncture site is dry*
- *Avoid a probing, traumatic venipuncture*
- *Avoid prolonged tourniquet application of first clenching*
- *Use correct size needle (~22gauge)*
- *Vacuum tubes should be filled completely*

Sampling methods has previously been mentioned as an important factor to consider. When working with serum, or plasma, it is worth considering that different hospitals may use different equipment and procedures leading to variations in samples. It is not without importance that the sampling method is

consistent throughout the study (unpublished observations). We recommend following the NIH guidelines for collecting serum and plasma. These guidelines ensure that samples are handled to ensure minimal contamination with cells prior to freezing of the samples. Sample collected in heparin-tubes are not suitable for qPCR profiling as heparin is a very strong PCR inhibitor, and is impossible to get rid of during RNA extraction.

A point that is often neglected to consider is the importance of considering whether the samples have been prone to haemolysis causing the presence of contaminating microRNAs from erythrocytes. Haemolysis can be detected either by spectrophotometry or by using a haemolysis indicating qPCR test (Blondal et al, 2013).

Another important recommendation is the use of carrier RNA in the extraction procedure. Due to the low total amounts of nucleic acids in the cell-free serum or plasma, the relative loss of microRNA due to adsorption to filters and plastic becomes high if no carrier is used (**Figure 15.10**). Using a carrier will not only give better yield, but also better reproducibility (Andreasen et al, 2010).

Second, our recommendations for reference genes in microRNA studies will always be to use stably expressed microRNAs rather than longer RNA. This becomes even more important when dealing with cell-free biofluids where the longer RNA (i.e. U6 or SNORNA) are perhaps not secreted nor protected to the same level as microRNA. microRNAs which are typically detected at medium to high levels in serum and plasma:

- → hsa-miR-103a-5p
- → hsa-miR-191-5p
- → hsa-miR-423-3p/5p
- → hsa-miR-451a

Finally, it is important to consider that serum and plasma contains high levels of PCR inhibitors, which may carry over during RNA extraction. For this reason, it is advisable to perform a serial dilution and test for signs of inhibition before increasing sample input amounts beyond the recommendations of the reagent provider. Also note that not all assays are equally prone to inhibition, so while some assays may seem unaffected by higher sample amounts, others may be severely inhibited in the same sample.

Recommendations for working with serum/plasma can be found at www.exiqon.com/serum-plasma-guidelines.

"Use carrier in low-content-samples:

urine, CSF, serum and plasma"

Urine, CSF and other biofluids

When working with biofluids such as urine or CSF it should be noted that these are even more dilute in RNA content than is the case for serum and plasma. Thus the use of a carrier in the sample preparation becomes even more important. Isolating exosomes before RNA extraction may help to increase yield these sample types. Furthermore, they too may contain PCR inhibitors, which should be tested for.

Finally it is worth noting that for urine, the concentration of RNA, cells, and other components may vary highly from sample to sample, depending on the time of day and state of hydration as well as disease state. This make normalization quite challenging, and it may be worth considering using the creatinine level or urine volume as a normalizer.

Learn more about microRNA in urine, CSF and exosomes

Watch the webinar: microRNA in biofluids and exosomes

Learn about microRNA in biofluids and biofluid sub-compartments like exosomes, their potential as biomarkers and how to isolate and detect them reliably. Results from a clinical study on microRNA biomarkers in biofluids will be presented

Poster presentation: microRNA in exosomes – robust biomarkes for disease

Learn how to isolate and profile exosome microRNAs from common biofluids. Valuable tips on how to set up experiments and see an example of how microRNA profiles from urine can be used to identify the various stages of prostate cancer.

Read about identification of biomarkers in urine.

Normalization and data analysis

1. **Interplate calibration**
2. **Choosing analysis software**
3. **Making biological sense of your data (miRSearch)**

The purpose of most microRNA qPCR studies is to look for biological differences. However, these may be masked by technical variation. The purpose of normalization is to filter out this technical variation, thus making the true biological differences more obvious. As described above, technical variation may arise from the differences in sample collection and handling, RNA extraction procedure and resulting RNA quality.

Interplate calibration

Some types of real-time PCR instruments, such as Roche LC480 which uses the 2nd derivative method for Cq calculations, give very reproducible results from run to run. In our hands, there is no need for interplate calibration when using the LC480. However, many qPCR cyclers do exhibit technical differences in amplification signal from run to run – particularly when the Cq values reported rely on baseline and threshold calculations. For this reason, it is often advisable to have all replicates and samples for one gene located on the same plate. However, in large studies with many samples and/or many microRNAs of interest (e.g. Exiqon's pre-designed human and rodent panels), this is not feasible. Instead, inter-plate calibration can be applied, where the Cq values across plates are calibrated to a reference with identical expression in each plate. Such an interplate calibrator (IPC) is present in triplicate on all Exiqon's ready-to-use panels in the miRCURY LNA™ Universal RT microRNA PCR system (pre-designed as well as

the flexible and fully custom designed Pick-&-Mix panel). Each IPC well contains both the detection primer set and the DNA template (making amplification independent of the sample). The IPC is present in triplicate in order to avoid losing an entire plate of data due to e.g. a pipetting error into an IPC well. In addition, we have placed the three IPC replicates in unique patterns for each of our plate types, making it possible to identify the plate used by its IPC pattern. Interplate calibration can be performed with the formula in the textbox.

Formula used by GenEx for interplate calibration:

$$Cq_{norm} = Cq_{GOI} - \frac{1}{m}\sum_{j=1}^{n}\left(Cq_{IPC_j} - \frac{1}{n}\sum_{i=1}^{n} Cq_{IPC_i}\right)$$

where GOI is the gene of interest, m is the number of interplate calibrators in run "m", and n is the total number of interplate calibrators.

For users of Exiqon's GenEx qPCR analysis software, interplate calibration is integrated in the pre-processing steps described in the GenEx qPCR analysis software guide.

Normalization to global mean

If a large screening (typically with >100 microRNAs) is performed with no previous knowledge of differential expression, it is expected that the majority of microRNAs will not be regulated between the samples. Thus will outweigh regulated microRNAs in terms of stability. Overall, individual differences in expression will be evened out in the mean of expression levels. The preferred method for normalization would be to the global mean (Mestdagh et al. 2009).

Be aware that in sample types where the general microRNA level has changed, global mean normalization may not be recommendable. An example of such a case could be serum or plasma with haemolysis, where the overall microRNA expression level has changed compared to a sample with no haemolysis (Blondal et al, 2013).

Normalization to reference genes

In studies with few microRNAs, one or more stably expressed endogenous control can be used. The preferred method for choosing these is based on a screening study where they showed

stable expression with the same behaviour as the global mean of all expressed microRNAs (Mestdagh et al. 2009; Chang et al, 2010).

Listed below are features that characterize a good endogenous control candidate for normalization of microRNA quantification:

- → Expression at similar level to the microRNAs of interest in the study
- → Invariant expression across all samples in the study (i.e. not regulated under the experimental conditions)
- → Similar small size as the microRNAs (i.e. similar stability, extraction and quantification efficiency)

If no screening study precedes the study, the above described method for reference gene selection cannot be applied. In this case, reference genes can be chosen based on literature. It is important that the reference genes are empirically validated for each study using tools such as NormFinder or GeNorm (part of various analysis software packages). No single reference gene can be recommended for use across all types of tissue and cells. Even among the most commonly used reference genes (e.g. beta-actin and GAPDH for mRNA expression) significant variations in expression level between samples can be observed (De Kok et al, 2005).

Generally, it is found that evaluation of several candidates is necessary in order to find the most appropriate reference gene(s) for each microRNA quantification study. If no previous data is available from the samples in question, it is recommend testing 5-6 different candidates. From these, normalization to 2-3 stably expressed genes, preferably microRNAs, would typically be sufficient.

"Use stably expressed microRNAs as endogenous references"

Depending on the origin of the sample, some commonly used reference microRNAs include: hsa-miR-103, hsa-miR-423-3p, hsa-miR-191, hsa-miR-16, hsa-miR-423-5p, and hsa-let-7a (Bargaje et al, 2010; Chang et al, 2010; Liang et al, 2007; Peltier and Latham, 2008).

Special care should be taken when choosing reference genes for biofluid samples with extracellular microRNAs. These sample types are virtually cell free, and thus RNA species expressed only within cells or released as degradation products (such as U6, 5S rRNA, snoRNAs etc.) will not be appropriate (see section regarding serum/plasma samples). Exiqon's guidelines for microRNA qPCR in serum/plasma discuss candidate microRNA

reference genes specifically for use with serum and plasma samples.

Choosing analysis software

If you are working with a limited number of assays combined with a relatively small number of samples, performing the data analysis in a spreadsheet is quite doable. However, as soon as the assay/ sample matrix starts growing, simple spreadsheet analysis becomes quite complex and it would be preferable to use a good data analysis software instead.

There is a number of different analysis software available on the market, and several of them are very good options. Before choosing which one to go with, you should consider not only price, but also how well the software integrates with the qPCR platform of your choice, the level of QC and pre-processing integrated in the software, and the downstream statistical methods available within the software.

With proper experimental set up, careful quality control and pre-processing of your data, generating the final results becomes easy when using a qPCR data analysis tool such as the Exiqon GenEx qPCR data analysis software. This qPCR software package makes it possible to perform statistical analysis and create publication ready figures with a few clicks and does not require any prior biostatistician skills.

Making biological sense of your data (miRSearch)

Once the samples have been prepared, qPCR run, and data analysed, it becomes time to make biological sense of the data. There are several online databases and tools available for microRNA. Exiqon have combined cross-annotated the major databases including Ensemble and miRbase to provide for seamless searches the best ones into one simple, easy-to-use tool named miRSearch. The tool is freely available regardless of platform used in the analysis, and is directly integrated into the Exiqon GenEx qPCR data analysis software. You may also use XploreRNA which is the App version of miRSearch.

Section 7

Concluding remarks

It is clear that setting up qPCR experiments properly is of critical importance for the success of the study. As described in this chapter a proper microRNA qPCR experiment requires profound understanding of the qPCR technique, the particular advantages and challenges of microRNA, the sample type to be studied, and the purpose of the study as well as careful consideration of each step of the experiment in terms of selection of sample size, replicates and the role of normalization.

By following the MIQE guidelines combined with the tips presented here, you will have a good foundation for setting up your own microRNA qPCR study properly and obtaining high quality data. Choose your experimental setup wisely and you have taken the first step towards the next big microRNA breakthrough.

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Chapter 16

EIGHT YEARS LATER: WHAT DIGITAL PCR OFFERED TO SCIENCE?

Afif AbdelNour

The Holy Spirit University of Kaslik

Section 1

Introduction

For the past 8 years digital PCR (A.K.A. dPCR or ddPCR) shaped the world of polymerase Chain Reaction (PCR). dPCR is considered to be the 3rd generation of PCR after the PCR and the qPCR. In this chapter we are going to shed the light on what dPCR offered to the scientific community and how this trend will evolve in the future.

Below is the overview of this chapter:

- History of dPCR by Alexander A. Morley

- Is dPCR a global technique?
- Will its use continue to expand exponentially?
- Does dPCR allow to publish better?
- dPCR usage trends

Mapping

Section 2

History of dPCR

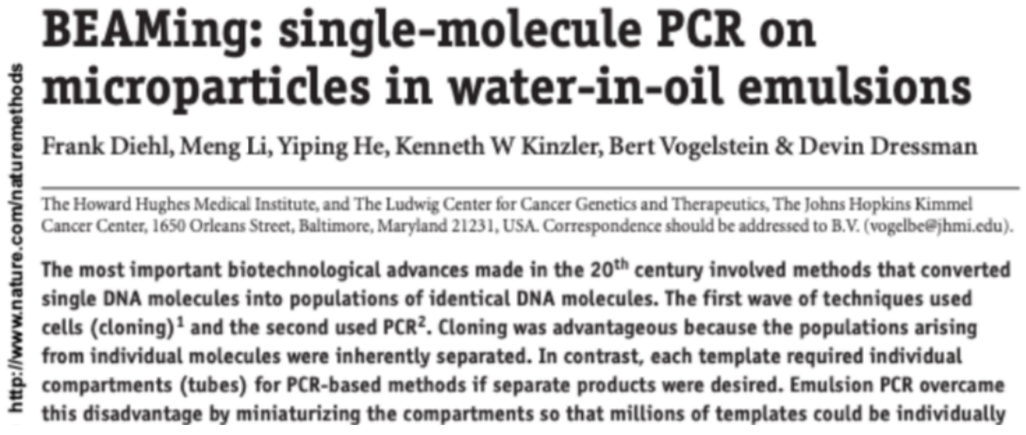
In 2014, Morley A.A. [1] published a very interesting paper tracing the beginnings of dPCR. To our surprise dPCR was invented before qPCR. The term “limiting dilution PCR” was first used in 1988 by Saiki et al.[2].
The first targeted virus to be detected by dPCR was HIV [3]. Around a dozen of papers were published after that mainly in the areas of virology and lymphoid biology and neoplasia.

From Morley AA, we can tell the following key points:

- **dPCR was invented before qPCR**
- **Limiting dilution PCR was first used in 1988 (Saiki et al. Science)**
- **HIV target sequences were detected by dPCR in 1990 (Simmonds et al. Journal of virology)**
- **The publications are in the areas of virology and lymphoid biology and neoplasia**

After that it was the void, qPCR was the trending technique monopolizing all researches done in the molecular biology labs.

In 2006, the team of Prof Vogelstein used the term BEAMing which stands for beads, emulsion, amplification, magnetics. A highly sensitive digital PCR method that combines emulsion PCR and flow cytometry to identify and quantify specific somatic mutations present in DNA [4, 5].



http://www.nature.com/naturemethods

BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions

Frank Diehl, Meng Li, Yiping He, Kenneth W Kinzler, Bert Vogelstein & Devin Dressman

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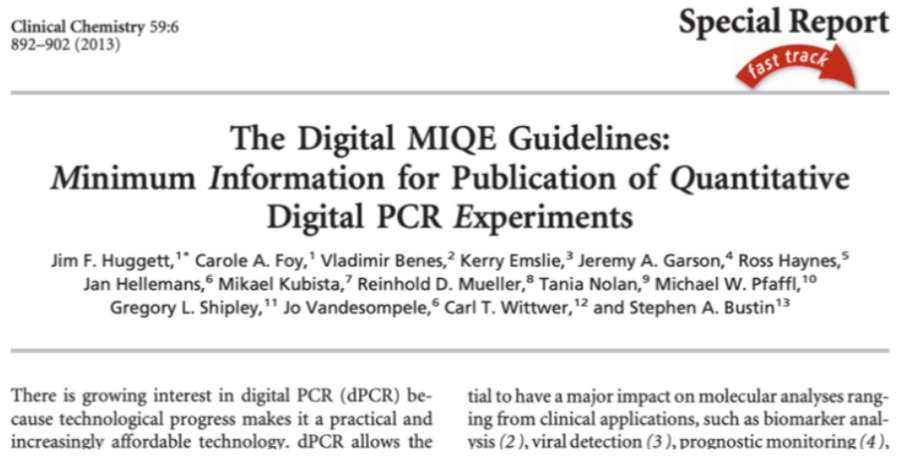
The most important biotechnological advances made in the 20th century involved methods that converted single DNA molecules into populations of identical DNA molecules. The first wave of techniques used cells (cloning)[1] and the second used PCR[2]. Cloning was advantageous because the populations arising from individual molecules were inherently separated. In contrast, each template required individual compartments (tubes) for PCR-based methods if separate products were desired. Emulsion PCR overcame this disadvantage by miniaturizing the compartments so that millions of templates could be individually

Companies followed the lead and we witnessed the innovation of many instruments starting by Fluidigm and ending with the new player in the dPCR arena, Dropworks. The familly portrait is shown hereafter.

The maturation of this technique was achieved by the publication of the digital MIQE guidelines [6]. As for the qPCR guidelines [7], the highest purposes of the dMIQE are to help scientific community to standardize experimental protocols, maximize efficient utilization of resources, and enhance the impact of this promising new technology. Allowing a reproducible experiments is a key point behind the dMIQE.



Clinical Chemistry 59:6
892–902 (2013)

Special Report

fast track

The Digital MIQE Guidelines: *M*inimum *I*nformation for Publication of *Q*uantitative Digital PCR *E*xperiments

Jim F. Huggett,[1*] Carole A. Foy,[1] Vladimir Benes,[2] Kerry Emslie,[3] Jeremy A. Garson,[4] Ross Haynes,[5] Jan Hellemans,[6] Mikael Kubista,[7] Reinhold D. Mueller,[8] Tania Nolan,[9] Michael W. Pfaffl,[10] Gregory L. Shipley,[11] Jo Vandesompele,[6] Carl T. Wittwer,[12] and Stephen A. Bustin[13]

There is growing interest in digital PCR (dPCR) because technological progress makes it a practical and increasingly affordable technology. dPCR allows the tial to have a major impact on molecular analyses ranging from clinical applications, such as biomarker analysis (*2*), viral detection (*3*), prognostic monitoring (*4*),

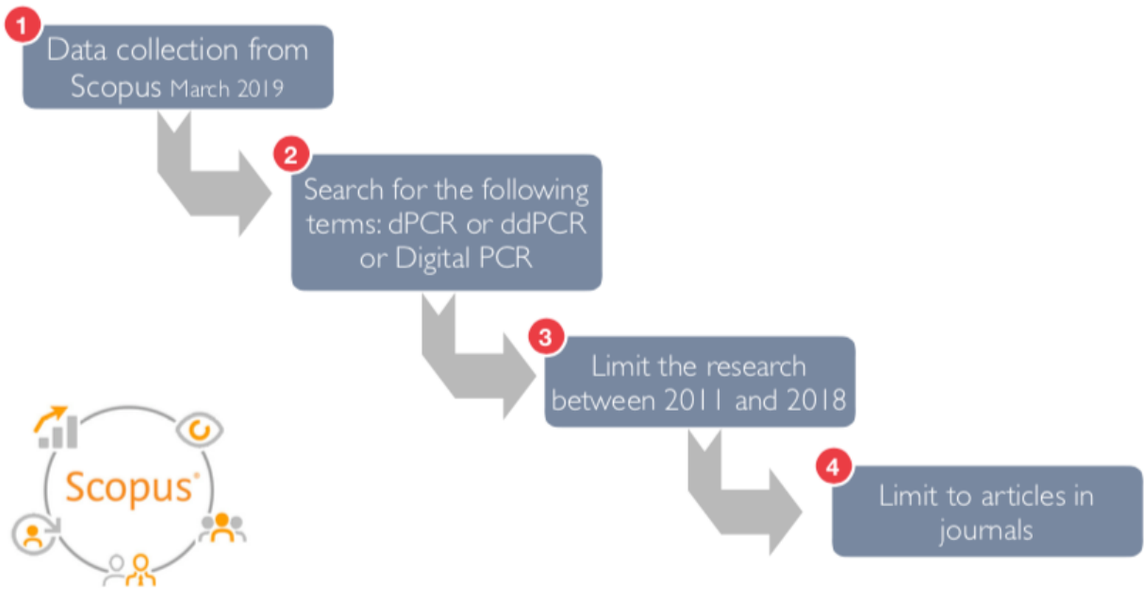
Section 3

Analysis of dPCR publications

1. Mapping of publications
2. Is dPCR a global technique?
3. Will its use continue to expand exponentially?
4. Does dPCR allow to publish better?
5. dPCR usage trends

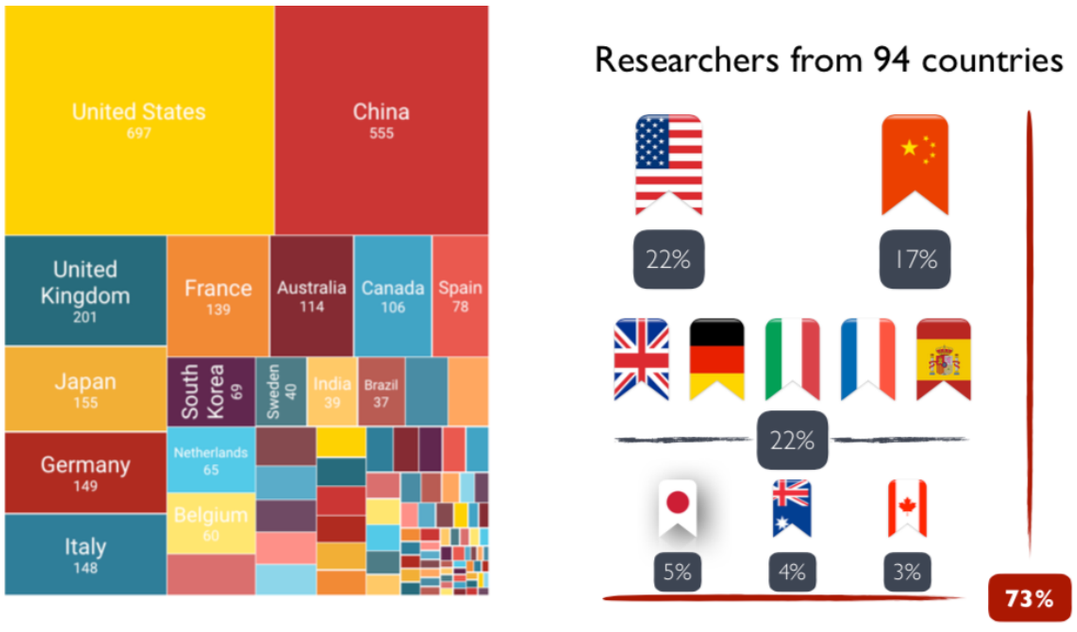
1 - Mapping of publications

Using the following terms: dPCR or ddPCR or Digital PCR, searched the Elseiver's powerful tool Scopus. We limited our search to articles published in journals between 2011 and 2018. We exclude posters, letters and reviews.



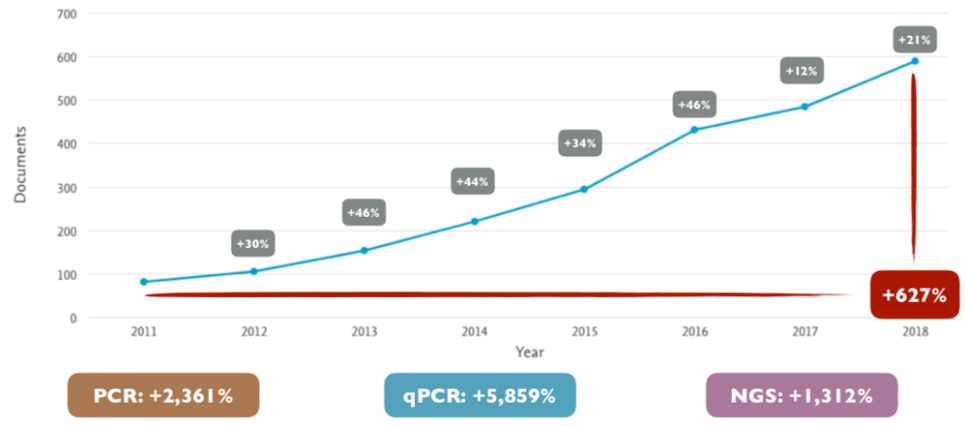
2 - Is dPCR a global technique?

Researches from 94 countries published at least 1 paper citing the dPCR. USA is first with 22% of the total papers followed by China (17%) and the big five European countries (UK, Germany, Italy, France and Spain) with 22%. To complete the the top 10, Japan (5%), Australia (4%) and Canada (3%) were added. Surprisingly 73% of the published papers were by this top 10 countries and the rest of the world only published 27%. Till today we can not consider the dPCR a global technique.



3 - Will its use continue to expand exponentially?

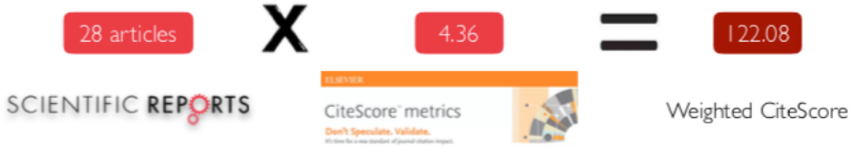
The mean growth of published papers citing dPCR was positive +33% with the highest in 2016 (+46%) and the lowest in 2017 (+12%). Although the growth between 2011 and 2018 was an astonishing +627% this remains far behind the growth of PCR, qPCR and NGS.
Unfortunately, dPCR technique did not expand exponentially.



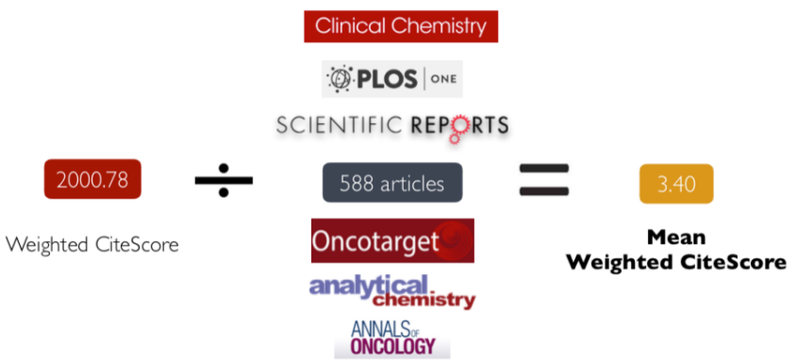
4 - Does dPCR allow to publish better?

To answer this question we first organized the papers according to the source journal. We obtained the CiteScores for each journal from Elseiver. And finally calculated the Weighted CiteScore per source and the mean CiteScore per year.

In 2018, 28 papers were published in Scientific Reports. The CiteScore for the later is 4.36, we multiplied the 28 by 4.36 in order to have a weighted CiteScore per source.

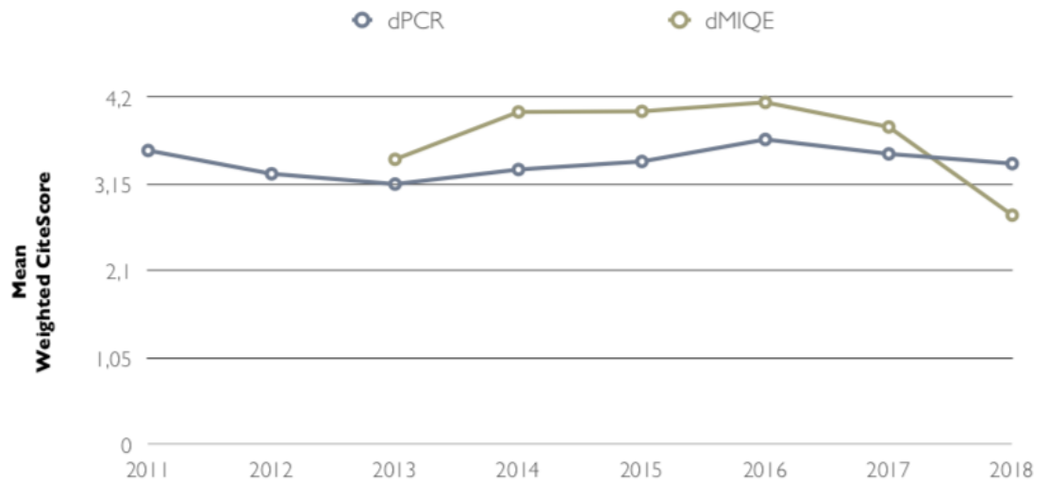


588 article were published in 2018 citing dPCR. We calculated the weighted CiteScore for every journal and we divided it by the number of published papers. By doing that, we obtained the mean weighted CiteScore per year.



This graph shows the mean weighted CiteScore per year. To be able to compare we added the mean weighted CiteScore per year (the same calculation method was used). Between 2011 and 2018 dPCR allowed researchers to published in a higher citescore journals compared to qPCR.

The dMIQE was published in 2013. between 2013 and 2017 researchers citing/using the dMIQE guidelines were able to publish in a higher CiteScore journals compared to dPCR. This trend was not respected in 2018 as for the first time dMIQE papers CiteScore was lower than the dPCR papers.



5 - dPCR usage trends

Using the same data base we were able to classify the published papers by area of usage. The trend was as follow:

1) Oncology 64%

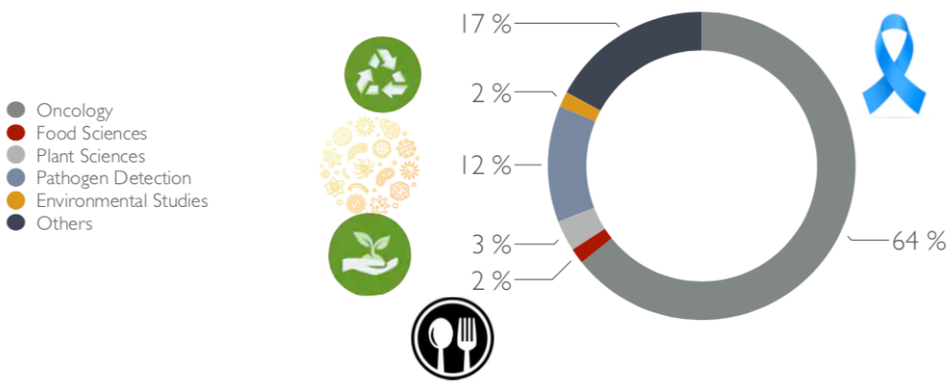
2) Food Sciences 2%

3) Plant Sciences 3%

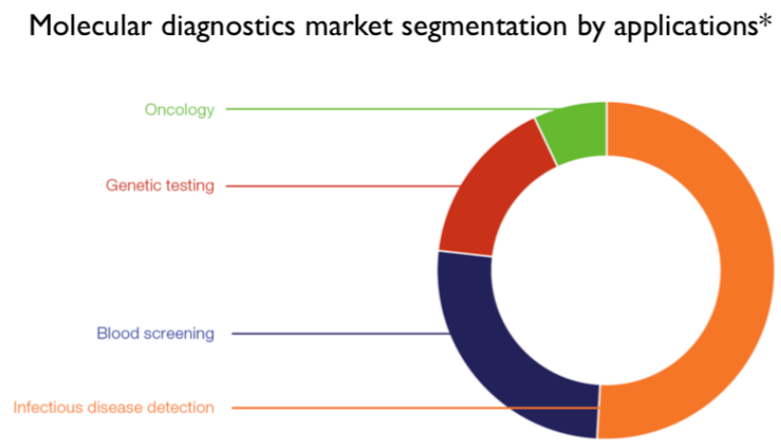
4) Pathogene Detection 12%

5) Environmental Studies 2%

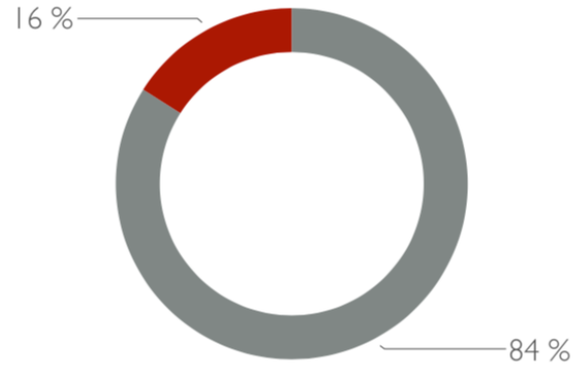
6) Others 17%



Regarding a specific focus on the molecular diagnostics, the market segmentation by applications in 2020 (forecast), showed that infectious disease detection needs were more than half of the total molecular diagnostic market. Oncology detection represents a small share of the market.



*Molecular Diagnostic Market - Global Forecast 2020



According to the two previous charts, in the past 8 years for every 1 paper published using dPCR and pathogen detection, at least 5 were published in the oncology field. So this imbalance with the market should be corrected first by the dPCR companies by proposing more solutions for the pathogen detection.

According to a recent review, few protocols detecting infectious diseases were developed for dPCR use [8]. It is worth mentioning that dPCR has several advantages in pathogen detection and quantification in comparison to qPCR (see figure below).

1. Hepatitis B (Hepatitis B virus)
2. HIV
3. Bacterial infections (Staphylococcus, Salmonella, and Listeria)
4. Tuberculosis (Mycobacterium tuberculosis)
5. Human herpes virus
6. Malaria (malaria parasites)

Advantages

- Absolute quantification (no need for a standard curve)
- Highly tolerant to inhibitors
- Compatible with difficult samples (blood)

Section 4

Conclusion

dPCR is still under used mainly for one and simple raison, the cost of the instrument. Compared to qPCR although the cost of an instrument was not far from 100,000 Euros in early 2000, this cost dropped drastically allowing researchers to perform more experiment and publish more. With the appearance of new companies the price for a dPCR instrument is decreasing, this will allow a more globalization of the technique.

Companies should focus on what the market needs, this should allow a more publication in the pathogen detection research/diagnostic field.

Although dMIQE is an essential guideline a new and ameliorated version of it should be drafted very soon.

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Chapter 17

Applying MIQE standards to dPCR experiments

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Section 1

Introduction

Quantification and characterization of nucleic acids has been a main area of research for several decades. Since its first description (Sykes et al., 1992), digital PCR (dPCR) has been the object of great interest and development. The principle behind dPCR is the division of a reaction into a large number of smaller homogenous reactions (or partitions) so that a number of the partitions contain no template. This is then thermocycled and read as individual partitions which will be classified as positive or negatives based on the presence or absence of amplified product. The outcome of the reading is a proportion of positives over total partitions, which can be converted to estimated copies using the Poisson distribution and the following equation.

$$\lambda = -\ln\left(1 - {}^{\mathbf{k}}/_{\mathbf{n}}\right)$$

Where k is the number of positive partitions and n the total number of partitions. This equation aims to account for the number of partitions that contain more than one molecule.

A main difference between digital PCR and qPCR is the fact that the quantification provided by dPCR does not rely on a calibration standard to assign a copy number. By comparison to a “relative” quantification method, dPCR performs an

“absolute” estimation, a measurement that can stand alone by itself without the need for a calibrator to which the result must be compared. The notion of “absolute quantification” should however not be mistaken for “perfect accuracy”. While the measurement provided by dPCR may not require a calibrator to obtain a numeric value, quantification can be influenced by factors such as the presence of PCR inhibitors or non-optimal thermal cycling conditions which can lead to a bias in result.

Today, several commercial platforms are available, with different specifications and characteristics. They can be classified in two main categories: chip-based dPCR and droplet dPCR. In chip-based dPCR platforms, the partition is done by compartimentalizing the reaction in wells of a micro-fluidic device or “chip” (Fluidigm, Life Technology, Formulatrix). In a droplet-based system (Bio-Rad, Raindance), a microfluidic circuit generates partitions in the form of micelles (or droplets), which remain in an emulsion state afterwards. Since the droplets can be recovered after thermocycling, these type of platforms can also lead to preparative applications of digital PCR.

This chapter will cover different applications of digital PCR, emphasizing the specificities of each, the differentiating factors form qPCR and the solutions and reagents that may exist for those specific uses. Additional information regarding the Minimum Information for Publication of Quantitative Digital PCR Experiments can be found at this link (Huggett el al, 2013). A more interactive platform for MIQE guidelines on qPCR and dPCR can be found under the form of a downloadable app.

Sample Acquisition, Handling and PCR Preparation

Although digital PCR has been shown to have an increased tolerance to some PCR inhibitors (Dingle et al., 2013; Racki et al., 2014), it is still susceptible to a range of inhibiting factors. It is for that reason that information on the method used for nucleic acid extraction/preparation is necessary and comprehensive inhibition controls used where a confident negative result is reported.

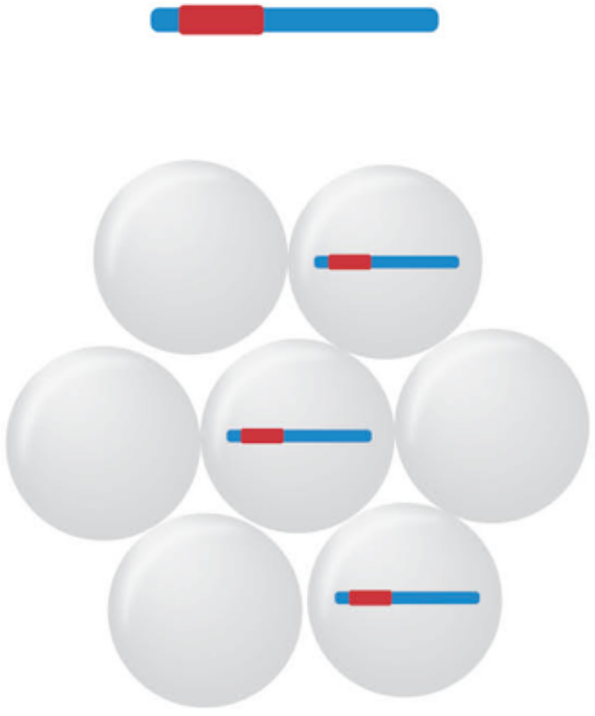
A description of the nature and handling of the biological sample is also necessary. Since dPCR is able to assess the physical linkage between targets, it is useful to have information on whether the original tissue was fresh/frozen or FFPE. The latter method introduces a large number of double strand breaks in the DNA, and would therefore be expected to affect the linkage results.

When using dPCR systems , it is important to report the solution in which the sample is resuspended. Some reagents such as detergents, which can be found in some direct lysis buffers, can interfere with the formation of droplets.

For dPCR to estimate template copies one of the assumptions is that DNA molecules will distribute across the partitions randomly. This assumption cannot be met if the molecules being quantified are linked in cis, as can be the case when tandem repeats of a gene need to be quantified. This is particularly important

when testing copy number variation (CNV) of certain oncogenes, and consequently the individual molecules need to be separates using methods like restriction digestion or preamplification. In such instances, it is necessary to report the detailed method(s) and parameters used, as well as what methods were used to evaluate the performance of the separation.

Figure 17.1 - Restriction digestion separates tandem gene copies

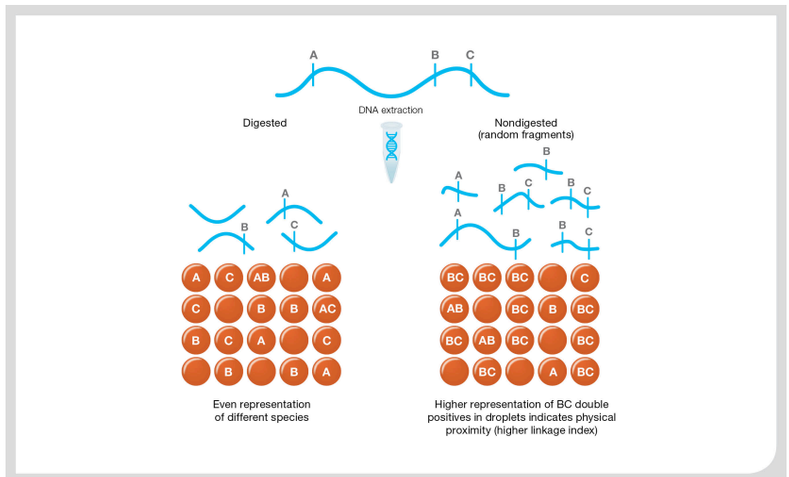


Single copy variant

Section 3

Linkage

One of the unique characteristics of digital PCR is to provide information on the physical linkage of two targets. By comparing the expected and observed proportions of double positive partitions, it is possible to determine whether the two genomic regions are on a same strand of DNA. An overrepresentation of double positive partitions would indicate that the two target region are linked, and this excess should increase as the distance between the targets (and therefore the chance of DNA breakage between them) decreases. This property of dPCR has been used to study the haplotypes of individuals and their association to clinical phenotypes (Roberts et al., 2014) and viral integration in host genomes (Sedlak et al., 2014).



Gene Expression and Digital RT-PCR

Due to the additional step of reverse transcription, quantification of RNA by qRT-PCR or dRT-PCR is subject to a greater risk of variation and bias. This risk can be mitigated by using one-step mixes (which diminishes the number of pipetting steps from sample to result) or, in some case, by increasing the number of replicates per sample. This will not control for bias in cDNA production. This can only be evaluated empirically and can differ significantly between both choice of enzyme of assay (Sanders et al 2013). Consequently RNA quantification by RT dPCR may actually need calibration to ensure reproducible results.

Another factor to keep in mind when testing RNA by RT dPCR is whether the reverse transcriptase used has RNAse H activity or not. An RNAse H positive enzyme will digest the RNA template in an RNA-cDNA heteroduplex, thereby increasing the chance that one RNA transcript will only lead to one molecule of cDNA. In RNAse H negative mixes, the same transcript may be read more than once, therefore leading to an artificial increase in the number of molecules. While this feature may be useful in cases where the presence of a rare transcript is normalized against a reference gene, it introduces an added uncertainty (were both RNAs reverse transcribed at the same rate?) and diminishes the accuracy of quantification for the original molecules.

Section 5

Rare Event Detection

One of the main applications of dPCR is the detection of very rare mutations where the wild type sequence predominates. A distinction should be made between the detection of unrelated targets (Rare Sequence Detection, e.g., a viral genome in a host DNA background) and the detection of related targets (Rare Mutation Detection, e.g., oncogenic mutations caused by SNP). While dPCR precision is paramount to quantification of both types of events at very low concentrations, the power of partitioning is especially useful for discriminating related sequences. While competition between the species can limit the detection of rare alleles to about 1%, digital PCR has been able to push this limit several logs lower. An example of detection at 0.0005% for a rare mutation and 0.0001% for a viral sequence can be seen at Ultra-Sensitive Rare Event Detection.

A. Results from 48 rare mutation detection test wells.

DNA	Positive Droplets	Negative Droplets	Tested Concentration (95% CI), copies/µl
Wild-type DNA	565,907	6,503	4,920 (4,890–4,950)
Mutant DNA	15	572,393	0.029 (0.017–0.046)
Ratio (mutant DNA/wild-type DNA x 100%)			0.00059%

B. Results from 24 rare sequence detection test wells.

DNA	Positive Droplets	Negative Droplets	Tested Concentration (95% CI), copies/µl
HHV-6 DNA	4	276,817	0.0159 (0.00479–0.0374)
Human genomic DNA (25-fold dilution)	7,730	4,469	1,100 (1,080–1,130)
Human genomic DNA (calculated)			27,500 (27,000–28,250)
Ratio (HHV-6 DNA/ human DNA x 100%)			0.000058%

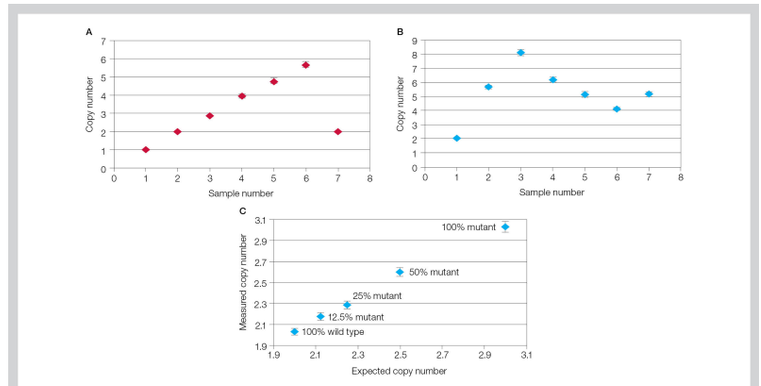
CI, confidence interval; HHV-6, human herpesvirus 6.

Results from test wells. A, detection of 0.0005% *BRAF V600E* mutant DNA in wild-type background; B, detection of human herpesvirus 6 in human genomic DNA background (less than one in a million).

In rare mutation detection studies (such as detection of Single Nucleotide Polymorphisms), the design of the assay and particularly of the probes is essential to the success of the experiment. While partitioning the reaction greatly helps in reducing the competition between products, the probes still need to be able to discriminate them based on a single nucleotide difference. Guidelines have been published in order to design successful assays (Droplet Digital PCR Application Guide, Chapter 2), but for many frequently studied mutations, commercial assays are available (Digital PCR assays).

Copy Number Variation

Another field that has greatly benefitted from digital PCR unparalleled sensitivity is the study of copy number variations (CNV) in the genome (Whale et al., 2012). While qPCR requires high numbers of replicates in order to reliably resolve 2 vs 3 copies per genome (50% difference), dPCR is able to distinguish 5 vs 6 copies per genome in a single well (High Resolution Copy Number Variation Analysis). It even enables users to detect increases in copy numbers in heterogenous samples (see table below). As mentioned before, one key aspect of CNV testing by dPCR is insuring that the targets are physically independent. The recommended approach to achieve this is a enzymatic digestion. This can be performed prior to adding the sample to the PCR mix or digestion can be performed in the PCR mix itself (Droplet Digital PCR Application Guide, Chapter 2).



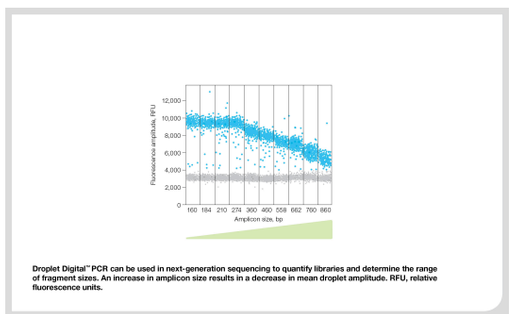
Determination of copy numbers. Determination of copy number for *MRGPRX1* (A) and *CCL3L1* (B) in homogeneous samples using the QX100™ Droplet Digital™ PCR (ddPCR™) System; C, determination of copy number for *CYP2D6* in a heterogeneous sample using the QX100 ddPCR System.

While the design of assays for CNV analyses does not present the same difficulties as in Rare Mutation Detection experiments, it is important to choose a reference

gene whose copy number will remain constant. Choosing a reference in a different chromosome is recommended, but in cases where the extent of the genomic anomalies is unknown (several polyploidic chromosomes or regions), the use of more than one reference per target can increase the confidence of the CNV determination. For this type of studies too, assays have been developed, validated and are commercially available (Digital PCR assays).

NGS Library Quantification

In the NGS (Next Generation Sequencing) workflow, quantification of the library to be read is a crucial step. Loading the system with too much material can lead to a large proportion of unusable data, whereas loading too little material will result in a waste of expensive consumables, sample and time. Several methods have been developed to quantify libraries, by direct spectrophotometry, using intercalant fluorophores or by qPCR, but recent research points toward a more reliable and sensitive quantification by digital PCR (White et al., 2009). Since the introduction of commercial digital PCR platforms, several solutions have been developed to quantify and characterize NGS libraries. The use of dPCR allows to not only obtain a precise quantification of the fragments in the library, but can also provide information on the quality of the libraries. The co-detection of two different probes reflects the appropriate ligation of the 5' and 3' adapters in the Truseq library, and the presence of high amplitude clusters is indicates the proportion of adapter dimers with no inserts (Quantification of NGS libraries by dPCR). The use of intercalant chemistry in dPCR (EvaGreen) was recently employed to assess the insert sizes in NGS libraries (Laurie et al., 2013).



Droplet Digital™ PCR can be used in next-generation sequencing to quantify libraries and determine the range of fragment sizes. An increase in amplicon size results in a decrease in mean droplet amplitude. RFU, relative fluorescence units.

Section 8

Diagnostic Applications

Although the commercial implementation of the technology is relatively new, dPCR is already being seen as a very promising development for clinical diagnostics. One of its main assets is the fact that it does not require a standard curve to assign a value. The concept of absolute quantification is extremely attractive, since it would reduce the intra- and inter-laboratory variability for a same assay. Several teams have already started to explore the use of dPCR for diagnostics of microbial infections (Kelley et al., 2013; Roberts et al., 2013), viral load (Henrich et al., 2012; Strain et al, 2013) and cancer markers (Nadauld et al., 2012; Gevensleben et al., 2013). Other advantages of dPCR for clinical diagnostics are its increased precision for quantification of low-levels of targets and its sensitivity for detection of allelic variants or mutations in an abundant wild-type background.

An important consideration when developing dPCR methods for clinical diagnostics is the risk for amplicon contamination. This is frequently observed in laboratories where the same assays are used routinely. One important step to resolve problems of amplicon contamination is to use dUTP in the PCR mix. The inclusion of dUTP in the amplicons sequence will render them sensitive to digestion by the UNG enzyme (uracil N-glycosylase). A pretreatment of the samples by UNG will remove contamination by pre-existing amplicons, minimizing the risk of false-positives in the workflow.

Acknowledgements

We sincerely thank Dr Jim Huggett for his help in reviewing this document.

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Chapter 18

Reproducible and Sensitive Assays using 3- and 6-colour Crystal Digital PCR™ for Detecting Point Mutations in Human Breast Cancer

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Section 1

Introduction

A brief introduction to the Naica™ System

The Naica System is a digital PCR platform launched in 2016 and consists of the Naica™ Geode and the Naica™ Prism3 instruments, as well as Crystal Reader and Crystal Miner software applications and a single consumable, the Sapphire chip. The features of the Naica System include, but are not limited to, compatibility with a wide range of fluorophores (FAM, Yakima Yellow, Cy3, HEX, ROX, Cy5, etc.), shortest time to result (2h 30 min), easy data visualisation and interpretation, and user-friendly workflow. The Sapphire chip, engineered using microfluidic technology, consists of four microfluidic wells where the PCR reaction sample is divided into up to 30,000 distinct partitions, called droplets, arranged in a monodispersed stable and homogenous crystal layer. This partitioning takes place in the Naica Geode. The number of nucleic acid molecules encapsulated in a droplet follows a Poisson law distribution (see here for Poisson Law), resulting in zero, one or more target molecules per droplet. The utilization of Poisson law for digital PCR is based on the critical assumption that all droplets are homogenous and monodispersed (Pinheiro and Emslie 2018). The Sapphire chips are then imaged by the Naica Prism3 and Crystal Reader software and further analysed using the Crystal Miner software for an easy-to-assess, transparent data output. The Naica System provides automated data processing and the possibility for manual adjustment pertaining to droplet detection and fluorescence thresholding.

A descriptive visual of the steps involved in Crystal digital PCR using the Naica System is available here: https://youtu.be/YYDhRCIPHNk.

For a brief overview of the principle of digital PCR and its applications, please see this video: https://youtu.be/Qqdmw3wvMFo.

The Crystal Miner software is intuitive and has a user-friendly interface, allowing users to dynamically visualise droplets in both crystal images and fluorescence dot-plots including 1-3 dimensional graphs. The image processing algorithms include a neural network that automatically excludes artefacts and non-homogenous droplets in crystal images with high reliability. The software also empowers users to accurately analyse the data using adapted statistical methods.

The currently available digital PCR system is the 3-colour Naica System that can detect targets in 3-colour channels and can be easily adapted to various digital PCR applications (Figure 1). A prototype of the Naica System that has the capacity to detect targets in 6-colours was successfully used in a proof-of-concept study for the identification of Epidermal Growth Factor Receptor (*EGFR*) sensitizing and resistance mutations in non-small cell lung cancer (NSCLC) patients (Madic et al. 2019). The Naica System 6-colour solution will be launched soon.

Figure 1. The commercially available Naica System, composed of the Naica Geode, the Naica Prism3 and the Sapphire chips

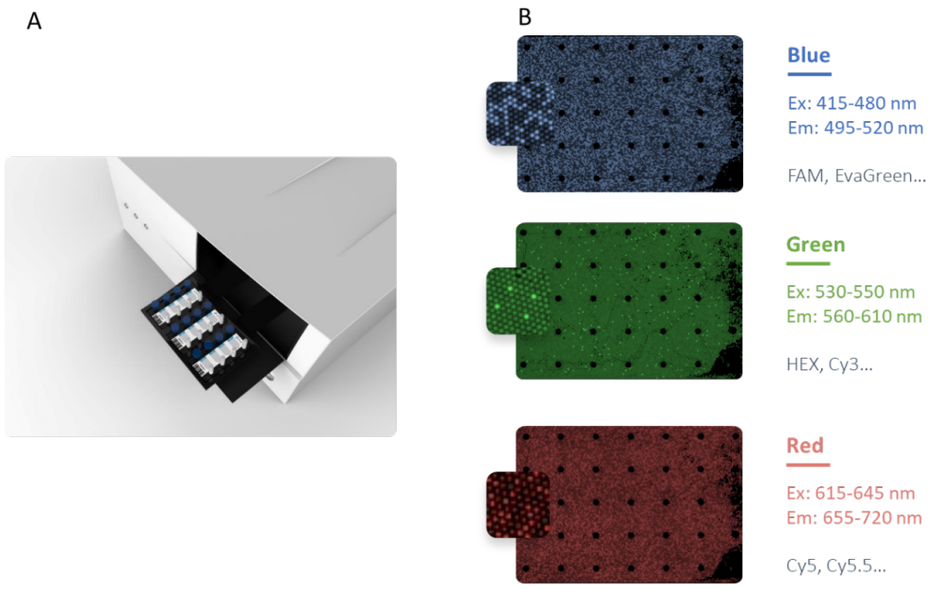


Figure 2. A) The Naica Prism3, a fluorescence reader for crystal droplets, and B) The three detection channels for targets and a few examples of the fluorophores that can be used.

Introduction to Naica System applications

Crystal Digital PCR has many advantages over traditional qPCR, including the lack of reliance on a standard curve, the user-friendliness, as well as robust and specific detection of rare events in a high background of wild-type (WT) DNA.

The Naica System has been optimized for applications in a wide range of nucleic acid quantification and analyses such as molecular and clinical oncology, molecular diagnostics, gene therapy, epigenetics, food safety monitoring, environmental testing, microbial ecology, and infectious diseases. The potential applications include copy number variation, rare event detection, gene editing, whole genome amplification, droplet recovery for downstream applications, NGS library quantification and NGS result validation.

One of the most useful applications of the Naica System is for accurately detecting rare events/mutations in various cancer types. In the case of NSCLC, *EGFR* activating and resistance mutations comprise the most prevalent mutations. Three and six-colour multiplex assays were designed to detect multiple mutations in a single assay, allowing to rapidly predict patient responses for standard therapy (tyrosine kinase inhibitors). Using a three-colour multiplexing assay, activating *L585R*, *L861Q* and multiple *EGFR* exon 19 alterations, together with the T790M resistance mutation and *EGFR* WT DNA in circulating tumour DNA (ctDNA) NSCLC patient samples were detected using two multiplex assays. The assay design and results are described in detail here: https://www.stillatechnologies.com/color-crystal-tm-digital-pcr-assays-for-egfr-mutation-detection/. Furthermore, using 6-colour multiplexing, a single assay was designed and validated to detect the 19 most prevalent sensitizing and resistance *EGFR* mutations, simultaneously quantifying five sensitizing mutations, 12 indels in exon 19, two resistance mutations and an internal amplification control in tumour tissue and cell-free DNA (cfDNA) from NSCLC patients. To

simultaneously detect the 12 most prevalent *EGFR* exon 19 alterations using only two probes, a drop-off assay composed of a drop-off probe spanning the genomic *EGFR* mutation hotspot and a reference probe spanning an adjacent *EGFR* WT genomic region was employed. For more details, please see https://www.ncbi.nlm.nih.gov/pubmed/30647840 (Madic et al. 2019).

Drop-off assays to detect the most prevalent colorectal carcinoma, *NRAS* and *KRAS* proto-oncogene mutations were also developed. The employment of a single amplicon and a limited number of probes to detect any genetic alteration occurring in a short genomic interval allows a rapid and a cost-efficient screening compared to conventional assays that would require many more probes to be designed and optimized. The drop-off assay is especially beneficial for sensitive and robust screening and therapeutic monitoring of clinical samples. The design and results of these three different drop-off assays are described here:

https://www.stillatechnologies.com/drop-off-crystal-dpcr-nras-kras-egfr-mutations/.

A complementary technical note detailing the use of the proprietary Crystal Miner software for the analysis of drop-off assays and a step-by-step guide for drop-off quantification can be found here: https://www.stillatechnologies.com/how-to-quantify-drop-off-digital-pcr-assays-with-crystal-miner/

Another advantage of digital PCR over traditional qPCR is that it is less sensitive to the presence of substances naturally present in sample extracts that can inhibit a PCR reaction. Indeed, many chemicals and substances can act as inhibitors in PCR-based reactions, which may be co-purified with the sample DNA and interfere with the PCR reaction. The presence of inhibitors in a sample can be a limiting factor in obtaining accurate or reliable results using qPCR. By quantifying and comparing the effect of two commonly detected inhibitor molecules, Humic Acid (found in soil samples) and Heparin (often used as a blood anticoagulant), in qPCR vs Crystal Digital PCR assays, it was observed that digital PCR could withstand up to twice the concentration of the inhibitors in the reaction mix as compared to qPCR. For details of the experimental results and more information, see here: https://www.stillatechnologies.com/effect-of-inhibitors-crystal-dpcr-vs-qpcr/

In addition to being compatible with TaqMan™ based PCR detection assays, Crystal Digital PCR is compatible with the use of EvaGreen® dye, an inert, non-toxic DNA-binding dye that becomes highly fluorescent when bound to double-stranded DNA (dsDNA) in a sequence-independent manner. EvaGreen offers the advantage of cost-effective probe-less detection using sequence-specific primers. EvaGreen dye can be used with Crystal Digital PCR for absolute quantification at a detection sensitivity of 0.2 copies/µl. For more information regarding EvaGreen dye

compatibility with the Naica System see: https://www.stillatechnologies.com/crystal-digital-pcr-using-evagreen/

To ease the transition and augment the adaptability of Crystal Digital PCR to different applications, the Naica System's droplet recovery feature allows the users to recover the droplets for downstream applications such as Next-generation Sequencing (NGS), nest PCR and gel electrophoresis. Users can perform an easy-to-follow protocol described here for recovering the samples:

https://www.stillatechnologies.com/droplet-recovery-with-crystal-digital-tm-pcr/

With recent advances in PCR detection technologies, nucleic acid quantification has now become a standard technique for genetic testing and analysis. In addition, precise and robust genetic testing has opened new frontiers for research and analysis in both food and environmental sample quality monitoring fronts. Digital PCR has been successfully used for detecting genetically modified crops and the composition of food items by several researchers worldwide. A step-by-step detailed tutorial on how to accurately quantify the presence of antibiotic resistance in solid organic waste from wastewater treatment plants is detailed here: https://www.gene-pi.com/tutorial/introduction-digital-pcr-using-evagreen-as-a-reporting-dye-2/. Organic waste from wastewater treatment plants, such as animal manure and sewage sludge, is known to be rich in antibiotic-resistant bacteria and antibiotic resistance genes. The utilization of organic waste for fertilization of the soil, thus, is a cause of concern as a potential source of infections and resistant genes. In this tutorial, ermB, a gene encoding erythromycin resistance, was detected in several organic waste samples that had previously been subjected to a cleaning process comprised of composting and digestion. A webinar titled "Identification of MRSA using 3-color Crystal Digital PCR" is available here: https://www.youtube.com/watch?v=jq-gUpFvhV8&feature=emb_title. Watch this webinar to explore how 3-colour Crystal Digital PCR can be employed for the detection of methicillin-resistant *Straphylococcus aureus* (MRSA).

For genetic testing and analysis in clinical settings, the amount of sample available is often limiting due primarily to low concentrations of target DNA. Additionally, for liquid biopsies (for example a blood draw or urine sample) and formalin-fixed paraffin-embedded (FFPE) samples, the DNA sample usually consists of a complex mixture of fragmented or ctDNA and WT DNA, demanding a highly sensitive quantification technique. By combining high sensitivity and the possibility to detect multiple targets in a single PCR due to its 3-colour detection technology, the Naica System ensures maximum information output from any precious sample. For example, quantification of Human Epidermal Growth Factor Receptor 2 (*HER2*) amplification, resulting from the overexpression of the *ERBB2* gene (also known as *ERBB2*, *CD320* and proto-oncogene *neu*) encoding the *HER2* receptor, is crucial for anti-*HER2* targeted therapy indication in

breast cancer patients. Current clinical diagnostic methods for *HER2* gene amplification evaluation in tumour samples are limited to immunohistochemistry (IHC), which may be complemented by fluorescence in-situ hybridization (FISH). However, these techniques are labour-intensive and prone to inter-observer variability (Rodriguez et al. 2016), and so require highly skilled personnel. To simplify the identification of *HER2* amplification in samples with a low tumoral fraction and render it resistant to observer-dependent techniques, a triplex Crystal Digital PCR assay was designed for *HER2* gene quantification. In addition to reducing hands-on time, this assay also allows distinguishing *HER2* amplification from polysomy on chromosome 17, a status that could lead to *HER2* status misinterpretation. DNA samples from seven breast cancer patients were analysed and the results were compared with standard diagnostic IHC and FISH procedures. For patients 1-4 and patients 6-7, the *HER2* positive and negative status, respectively, determined by Crystal Digital PCR was concordant with the IHC/FISH techniques. Additionally, Crystal Digital PCR detected polysomy on chromosome 17 in patient 6. Interestingly, for patient 5 using standard diagnostic procedures, amplification of *HER2* was detected; however, using Crystal Digital PCR the *HER2* elevation was attributed to chromosome 17 polysomy, a critical factor for the therapeutic consideration of breast cancer patients. Moreover, using this method, mutant fractions as low as 2% were detected, corresponding to an *HER2*/*TSN* (reference gene) normalized ratio of 1.2. Access the application note detailing the results described here: https://www.stillatechnologies.com/detection-of-her2-copy-number-variation-with-crystal-digital-tm-pcr/. Another valuable resource, a webinar, on liquid biopsy workflow and therapeutic monitoring by experts is available here: https://www.youtube.com/watch?time_continue=3&v=dTUUbfUG6mI&feature=emb_title

In the following section, we describe the advantages of multi-colour digital detection for monitoring common breast cancer mutations in liquid biopsy patient samples. Due to factors frequently limiting tumour tissue sampling such as tumour location, size and patient health complications, minimally invasive liquid biopsies represent a valuable source of ctDNA from which the genomic alterations of a patient's tumour can be determined. Indeed, tumours release nucleic acids (DNA or RNA) into the bloodstream, which can be recovered from plasma and used as a surrogate source of tumour DNA (C. Jovelet et al. 2016). This valuable source of genetic material requires sensitive and precise detection methods to quantify a maximum number of genetic alterations. Here, we present the Naica System in both 3-colour and 6-colour detection, as a fast and economical solution for reliable and robust quantification of liquid biopsy-sourced nucleic acid.

6- and 3-colour Crystal Digital PCR assays for screening genomic alterations in breast cancer

Breast cancer subtype characterisation

Breast cancer is the most prevalent cancer among women worldwide with an estimated 1.6 million cases diagnosed in 2012 and remains a major health problem (Ferlay et al. 2015). Breast cancer is a heterogeneous disease and can be categorized into various subtypes based on the expression of molecular receptors and have different prognosis and treatment (Koboldt et al. 2012):

1. Estrogen receptor-positive (ER+)/progesterone receptor-positive (PR+) breast tumours are responsive to endocrine therapies (such as tamoxifen and aromatase inhibitors). These types of hormonal therapies have improved patient outcomes in ER+ metastatic breast cancer (Kurosky et al. 2018).

2. *HER2* positive (HER2+) breast tumours are characterized by the overexpression of the *HER2* gene. It is detected in approximately 25–30% of breast cancers and is often associated with poor prognosis. Trastuzumab has been shown to be an effective treatment for HER2+ breast cancer (Slamon et al. 2011).

3. Triple-negative tumours, not displaying any of the three- ER, PR, and *HER2* within the tumour, are associated with high mortality rates and are not responsive to current targeted treatment approaches. The gold-standard approach for this subtype of cancer is systemic chemotherapy.

In breast cancer, the detection of *HER2* amplification in tumour-derived DNA can guide the therapeutic care of patients. IHC and FISH are currently the standard methods to assess *HER2* overexpression. However, nucleic acid quantification technologies, such as digital PCR are highly accurate methods to quantify DNA copy number and a robust solution for *HER2* diagnostics *MRM1*(Page et al. 2011).

With emerging technological advances, digital PCR has become a powerful tool to analyse liquid biopsy samples to quantify genetic alterations that can be associated with treatment decision and outcome. PI3K/AKT is one of the main pathways involved in the oncogenesis of breast cancer. *PIK3CA* is the gene coding for the enzyme (PI3K) playing a role downstream of *HER2*. Recurrent somatic mutations across all breast cancer subtypes were found in *PIK3CA* (36%) (Weisman et al. 2016). Detecting these mutations is important as targeted therapies exist and can improve the patient's Progression-Free Survival (PFS) (André et al. 2016). *PIK3CA* gene mutations are the most frequent events (40%) associated with hormonal therapy resistance in ER+ breast cancers. Mutations in *PIK3CA* generally occur in one of the two hotspot regions (exon 9: E545K or E542K, or exon 20: H1047R) (Araki and Miyoshi 2018). The early identification of *PIK3CA* mutations in patients undergoing hormone therapy could help better redefine the therapy. Moreover, most of the patients affected by ER+/HER2– tumours presented only bone metastases, a metastatic site that is associated with difficult pathological and molecular analyses. Detection of ctDNA in liquid biopsies is a promising alternative for the global evaluation of all metastatic sites because it eliminates invasive biopsy and the specific difficulties of less accessible bone metastases.

Early detection of relapse and residual disease

Collecting serial liquid biopsy samples to achieve longitudinal monitoring of tumour burden and tumour genetic heterogeneity is essential to evaluate the efficacy of a treatment. Indeed, levels of ctDNA have been shown to correlate with the overall survival of breast cancer patients and provide the earliest measurement of treatment response (Speicher and Pantel 2014; Yu et al. 2019). For example, a recent study examined ctDNA in early-stage before and after therapy and observed drug response showed that relative changes in mutated *PIK3CA* ctDNA levels can be used as a strong predictive tool of response to drug therapy (O'Leary et al. 2018)

Liquid biopsies are also emerging tools for the early detection of relapse in breast cancer patients. Moreover, minimally invasive ctDNA detection can be used to monitor post-operation patients for cfDNA analysis, to search for minimal residual disease (MRD) and to estimate the chances of recurrence. Studies have demonstrated that the follow-up monitoring of genomic alterations in ctDNA (*HER2* amplification, *PIK3CA* mutations)

enables physicians to detect relapse months before the detection by other follow-up methods including palpable, clinical symptoms and imaging (Olsson et al. 2015). In these studies, target mutations were first identified by tumour sample sequencing followed by ctDNA detection using droplet digital™ PCR (ddPCR™). They further used a digital PCR assay for the analysis of *PIK3CA* mutations in breast cancer patients at different stages of the disease (stages I to III). This study stratified the subjects into two groups with either high or low levels of ctDNA harbouring *PIK3CA* mutations, and subsequently concluded that a shorter recurrence-free survival was associated with the group with high *PIK3CA* mutations levels in plasma ctDNA (Oshiro et al. 2015).

Multiplex digital PCR assays for screening prevalent genomic alterations in breast cancer

The detection and clinical use of cfDNA have long remained challenging due to the complex mixture of a low level of tumour cfDNA and WT cfDNA. Highly robust and selective techniques are required to detect reliably mutations of clinical relevance. Digital PCR is a proven clinical tool as it combines high sensitivity and absolute quantification with a cost-effective short time-to-results. With the ability to partition samples into thousands of individual reactions, digital PCR enables superior target detection and quantification, allowing the accurate detection of rare mutations in low concentration liquid biopsy ctDNA samples (Madic et al. 2019).

Using the 3-colour Naica Crystal Digital PCR platform, a panel was developed for the detection of both *HER2* amplification and chromosome 17 polysomy in a single PCR. Furthermore, using the 6-colour Naica Crystal Digital PCR prototype system, the detection of *HER2* amplification and chromosome 17 polysomy was combined with the simultaneous quantification of the 3 most prevalent *PIK3CA* mutations in breast cancer samples all in a single reaction.

As previously described, the analysis of cfDNA in breast cancer enables the detection and monitoring of molecular abnormalities. The 3-colour and 6-colour assays could provide reliable early monitoring of treatment response and relapse (before other follow-up methods) and detection of resistance mutations. The 6-colour breast cancer panel described here in this chapter is the first digital PCR assay that combines both *HER2* amplification detection and the detection of the main *PIK3CA* mutations in a single assay, maximizing the information output from a single patient sample. Together, these genetic abnormalities account for more than 50% of breast cancers (Slamon et al. 2011; Weisman et al. 2016). The increased number of detection channels with this 6-colour detection platform, combined with the sensitivity of the Naica System, and a multiplexing strategy based on clinical

needs, advances the current limits of tumour genotyping and liquid biopsy-based breast cancer monitoring.

Assay design and performance evaluation

Design of a 3-colour HER2 digital PCR panel

A 3-plex digital PCR assay including primers and TaqMan probes was designed to detect both *HER2* amplification and chromosome 17 polysomy, a distinct condition where additional copies of chromosome 17 are present, leading to *HER2* status misinterpretation. *HER2* amplification is measured by calculating the ratio of the absolute concentration of the *HER2* gene on chromosome 17 over the absolute concentration of a reference gene, such as the Translin (*TSN*) encoding gene located on chromosome 2. For chromosome 17 polysomy detection, the ratio of Mitochondrial rRNA Methyltransferase 1 (*MRM1*) encoding gene concentration over *TSN* concentration is also measured. The *MRM1* gene is located on chromosome 17 and is not affected by *HER2* amplification but its copy number varies according to chromosome 17 polysomy. Primers for the amplification of *HER2*, *TSN* and *MRM1* (Figure 3) were selected to generate short amplicons of less than 100bp to ensure detection compatibility in fragmented DNA populations typical of ctDNA extracted from plasma or from FFPE tissues. The annealing temperature, number of cycles and primers concentrations were chosen to ensure optimal amplification, as scored by the distinction between positive and negative partitions, and to avoid nonspecific amplifications. TaqMan probes were labelled with a selection of fluorophores compatible with the Naica Prism3 detection channels: FAM, Cy3 and Cy5 for *HER2*, *MRM1* and *TSN* genes, respectively (Figure 4). The sensitivity of *HER2* copy number variation detection was measured on serially diluted samples where human genomic DNA was spiked with genomic DNA extracted from the SKBR3 cell line (*HER2*/*TSN* ratio of 10:1). SKBR3 fractions ranged from 3% to 13% and modelled a theoretical range of *HER2*/*TSN* ratios from 1.16 to 2.07. Prior to PCR, samples were fragmented using restriction endonuclease enzyme to ensure separation of the *HER2* copies and assayed in triplicate experiments.

Design of a 6-colour Crystal Digital PCR panel for the detection of HER2 amplification and PIK3CA point mutations:

To assemble the 6-colour Crystal Digital PCR panel, the 3-colour Crystal Digital PCR assay for the detection of *HER2* amplification described above was combined with primers and TaqMan probes targeting the most prevalent *PIK3CA* mutations in breast cancer (Figure 3). *PIK3CA* c.3140A>G (H1047R), c.1624G>A (E542K) and c.1633G>A (E545K). These mutations were detected using two primer pairs generating short amplicons (<78bp) and three TaqMan probes labelled with the ROX fluorophore. To increase mismatch discrimination, probe length was kept short (<19pb)

and a Minor Groove Binder (MGB) group was added to their 3' extremities. The WT *PIK3CA* allele was detected using a 19 base-pair TaqMan-MGB probe labelled with the Yakima Yellow fluorophore and targeting the c.3140A position. The remaining 6th detection channel was used for the detection of PhiX174 bacteriophage DNA as an exogenous internal control using a specific primer pair and an ATTO700 labelled TaqMan probe (Figure 3, Figure 4). The sensitivity of *HER2* amplification detection was evaluated using the 6-colour Crystal Digital PCR panel according to the method described above. *PIK3CA* mutation detection sensitivity was measured on serial dilutions of mutant DNAs in a background of WT DNA. The limit of blank (LOB) was evaluated by running 30 negative control replicates containing 104 copies of WT genomic DNA. For more details about setting the LOB and LOD for digital PCR assays, please see https://www.gene-pi.com/item/lob-lod-determination-2/. The decision tree to follow to determine the LOB while setting up an experiment can be viewed in this technical note: https://www.stillatechnologies.com/how-to-characterize-the-limit-of-blank-for-digital-pcr/. An in-depth explanation of the mathematical calculation involved for the determination of LOB is available here: https://www.gene-pi.com/statistical-tools/.

Figure 3: Localisation and design of the 3-colour and the 6-colour Crystal Digital PCR assays for the detection of *HER2* copy number variations and the 3 most prevalent *PIK3CA* mutations in breast cancer.

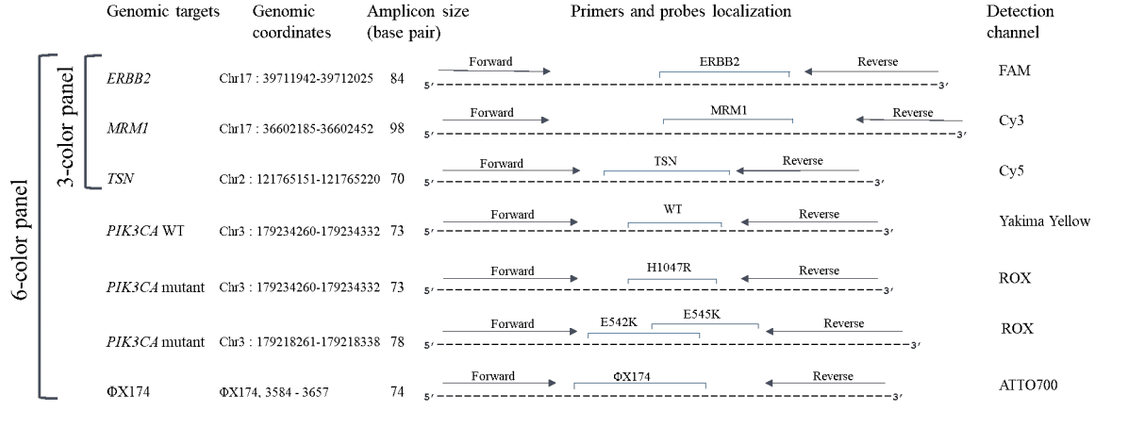
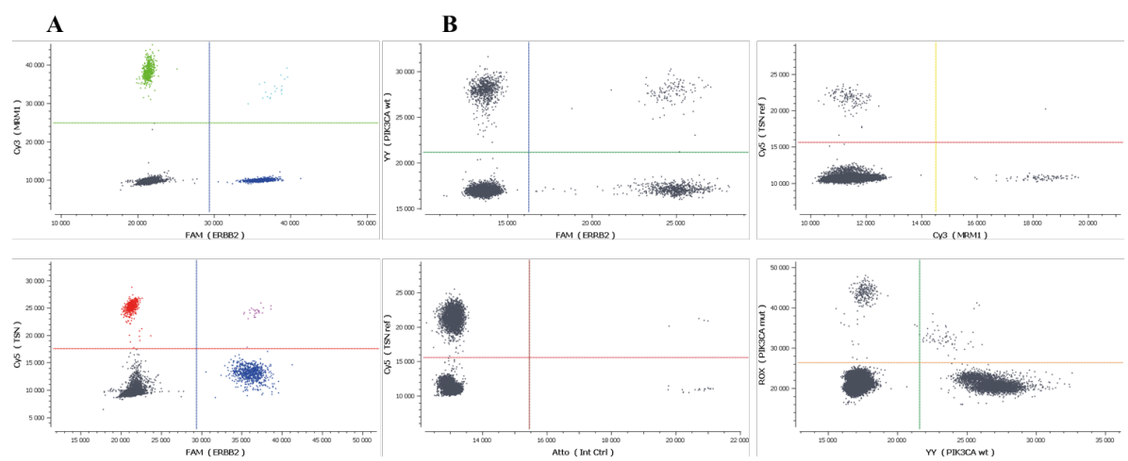


Figure 4: A) 2D dot plots of the 3-colour *HER2* digital PCR panel, and B) 2D dot plots of the 6-colour *HER2* digital PCR panel. Each target and its respective fluorescent reporter are indicated on the X- and Y-axes.



Results

Evaluation of the 6-colour Crystal Digital PCR panel on reconstruction experiments

Plasma DNAs extracted from breast cancer patients and healthy individuals were quantified and mixed to generate nine composed samples with varying *PIK3CA* mutant fractions and *HER2* copy number variation. The reconstructed samples were blindly tested in triplicate experiments each containing 10 µl of DNA and 500 copies of PhiX174 DNA. Positive and negative control wells containing a known concentration of mutant *PIK3CA* and WT commercial DNA, respectively, were included in each run.

Sensitivity of *HER2* amplification detection using 3-colour Crystal Digital PCR

The experimental *HER2/TSN* ratios were obtained by adding a mutant DNA bearing an *HER2/TSN* ratio of 10:1 to WT genomic DNA. Samples were assayed in triplicate using the 3-colour Crystal Digital PCR panel and absolute concentrations of *HER2* and *TSN* were calculated. To determine statistically the presence of DNA with *HER2* copy number variation with 95% confidence level (i.e. less than 5% false positives), Welch's t-test (Whale et al. 2012) was applied on the log-ratio *HER2/TSN* (approximated by a Normal distribution) in comparison with 36

negative control replicates. Using this method, a significant presence of mutant DNA in mutant fractions of 3%, corresponding to an *HER2/TSN* ratio of 1.16 was observed (Figure 5 A).

Sensitivity of detection of *HER2* amplification and *PIK3CA* mutations using 6-colour Crystal Digital PCR

The sensitivity of *HER2* amplification detection using the 6-colour Crystal Digital PCR panel was evaluated using the same approach as described in the 3-colour assays. The 6-colour Crystal Digital PCR panel enabled the detection of mutant fractions as low as 3%, corresponding to an *HER2/TSN* ratio of 1.16 (Figure 5 B). The three most prevalent *PIK3CA* mutations in breast cancer (*PIK3CA H1047R*, *E542K* and *E545K*) were detected in the ROX channel of the 6-colour Crystal Digital PCR panel. To calculate the LOB for mutant *PIK3CA* detection, the occurrence of false-positive events observed in negative controls was recorded and the mean (μ) of the false-positive distribution was calculated and corrected using the following formula:

$$\mu corr = \mu + 1.645\ \sigma / \sqrt{R}$$

Where σ is the standard deviation of this distribution and R is the number of negative control replicates. The LOB with a 95% confidence level was determined by fitting the calculated μcorr using Normal Law approximation and Chernoff's inequality. The LOB was thus determined as six false-positive partitions. The sensitivity of detection was determined for each *PIK3CA* mutation using a serial dilution of mutant DNA ranging from 500 to 5 copies per µl in a constant background of 104 copies of WT DNA per µl and representing a mutant allele fraction (MAF) ranging from 5% to 0.05%. Each dilution point was assayed in triplicate, except for the lowest mutant concentration that was assayed in quadruplicate. To determine whether the observed mutant quantity was significantly above the LOB, a Bayesian approach based on the probability of occurrence of false positives was used. For each result, the number of positive partitions was corrected by deducting eventual false-positive partitions weighted by their probability distribution. A sample was considered negative when the lower limit of the corrected 95% confidence interval included zero. According to this Bayesian correction, the limit of detection (LOD) at 95% confidence level was experimentally determined as 25 copies per 25 µl reaction for *PIK3CA H1047R*, and 5 copies per 25 µl reaction for *E542K* and *E545K*, which represents a minimum detectable MAF of 0.25% and 0.05% respectively in a background of 104 WT copies.

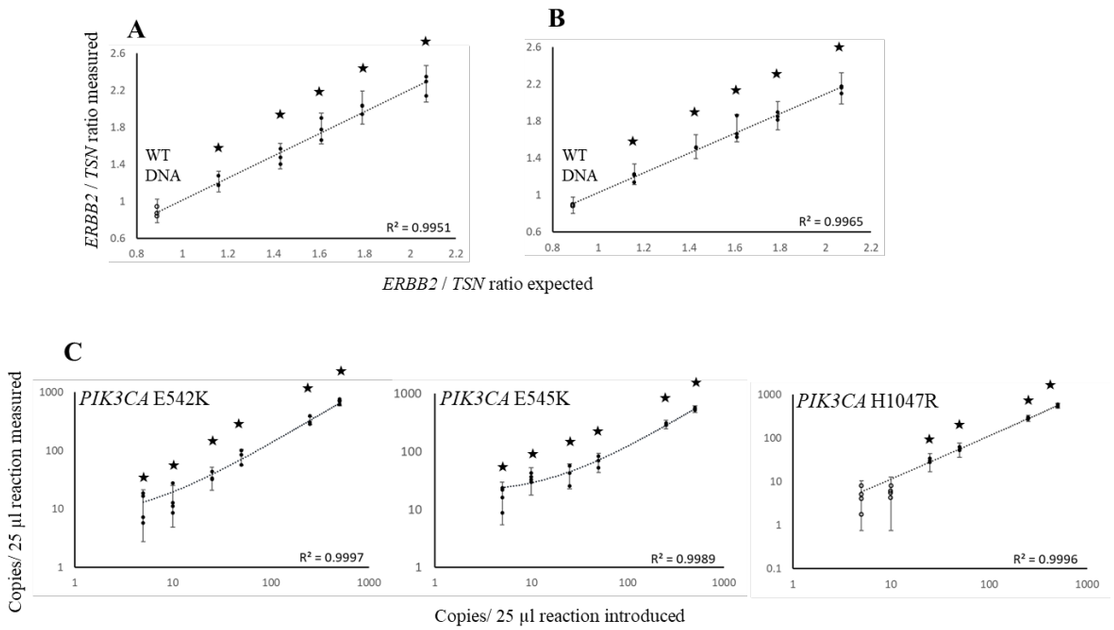


Figure 5: A) measure of different *HER2/TSN* ratios in reconstruction experiments using the 3-colour Crystal Digital PCR assay. B) measure of different *HER2/TSN* ratios in reconstruction experiments using the 6-colour Crystal Digital PCR assay. C) results obtained using the 6-colour Crystal Digital PCR assay on a serial dilution of mutant *PIK3CAPIK3CA* in a background of WT DNA (* indicates that detection is statistically significant, the theoretical 95% confidence intervals are represented as vertical bars, empty circles represent measures which are under the LOB).

Evaluation of the 6-colour Crystal Digital PCR panel on reconstruction experiments

Reconstructed samples (n=9) derived from plasma DNA from breast cancer patients and healthy individuals were assembled to obtain different concentrations of mutant *PIK3CA* and *HER2/TSN* ratios. The detection of *PIK3CA* mutations and *HER2* copy number variation was estimated using the statistical tools described earlier. To estimate the *HER2* copy number variation (Figure 5 B), 29 WT DNA controls extracted from plasma were used as controls. The results are shown in Table 1. Sample 9 containing 1.9 mutant *PIK3CA* copies per µl in a background of 348 WT DNA copies per µl (MAF of 0.55%) was detected as positive, whereas Sample 8 containing 0.9 mutant *PIK3CA* copies per µl in a background of 348 WT copies per µl (MAF of 0.26%) was found negative (Figure 5 C). Samples 3, 4 and 6 with expected *HER2/TSN* ratios of 1.55, 1.44 and 1.39 were detected positive after digital PCR and statistical analysis. Sample 5, with an expected *HER2/TSN* ratio of 1.34 was not considered positive as the lower limit of the 99.5% confidence interval calculated following its testing included zero. No false-positive mutant *PIK3CA* and *HER2* copy number variations were observed in WT DNA only samples.

Sample no	Mutational status of sample	WT DNA concentration (cp/µl plasma DNA)		*HER2/TSN* ratio			*PIK3CA* copies/µl plasma DNA		
		Expected	measured	Expected	Measured	Detected (CL >99.5%)	Expected	measured	Detected (CL>95%)
1	WT	115	92	1.2	1.2	no	0	0	no
2	WT	229	207	1.2	1.3	no	0	0	no
3	*HER2* amplification	229	205	1.55	1.55	yes	0	0	no
4	*HER2* amplification	229	211	1.44	1.56	yes	0	0	no
5	*HER2* amplification	229	208	1.34	1.42	no	0	0	no
6	*HER2* amplification	229	206	1.39	1.42	yes	0	0	no
7	WT	35	32	1.2	1.19	no	0	0	no
8	*PIK3CA* p.E542K	348	352	1.2	1.25	no	0.9	0	no
9	*PIK3CA* p.E545K	348	340	1.2	1.15	no	1.9	0.6	yes

Table 1: *HER2/TSN* ratio and *PIK3CA* mutations detected using the 6-colour Crystal Digital PCR panel in reconstructed samples derived from plasma DNA

Section 4

Conclusion

Digital PCR is a promising clinical tool as it combines high sensitivity and absolute quantification with a short time-to-results and a low cost. With the ability to partition samples into tens of thousands of individual reactions, digital PCR allows superior target detection and quantification, enabling accurate detection of rare mutations in low concentration liquid biopsy ctDNA samples and opening new clinical opportunities for this complex mixture of DNA. Here, the 6-colour Crystal Digital PCR technology enabled the monitoring of the main genomic alterations in patients with breast cancer in a first of its kind assay. *PIK3CA* mutations detected were not identified individually in this assay, instead were functionally grouped in separate detection channels based on the current knowledge about treatment efficacy prediction. Although this chapter details a panel for detecting major genomic alterations in breast cancer patients, it can also be used as a model for other oncology analysis developments. The 6-colour Crystal Digital PCR technology can be flexible and evolutive in terms of assay design. The increase in the number of detection channels and multiplexing strategy, combined with the high sensitivity of Crystal Digital PCR, represents a promising advance for genotyping and tumour monitoring in oncology.

Section 5

Advanced quality control using Crystal Miner

Software description

Crystal Miner is Stilla Technologies' proprietary software component of the Naica System. The Crystal Miner is designed for high-precision genetic data analysis based on Crystal Digital PCR. The Crystal Miner software is a user-friendly and transparent software. It is a powerful tool for statistical analysis of digital PCR data. Additionally, it allows users to visualise the droplets, select the desired droplet populations for analysis and produce reliable and accurate results.

The Crystal Miner software can be downloaded here: https://www.stillatechnologies.com/software/. The software also comes with demo data for first-time users to try different features and analyse data.

Unique characteristics and functions

The Crystal Miner software allows intuitive data visualisation and interpretation. The software offers the droplets to be distinguished in 3-colour channels, and this provides a wide spectrum for the users to visualise the droplets in. The droplets are detected based on the fluorescence emitted by the fluorophores upon hydrolysation or binding to dsDNA. Crystal Miner is compatible with a wide range of fluorescent dyes (non-exhaustive list: FAM, HEX, ROX, Yakima Yellow, Cy3, Cy5,

EvaGreen) and accurately detects the targets in the Blue, Green and Red detection channels (Figure 2). The user has the flexibility to choose from these fluorophores and select the ones that seem most compatible with their assay design. The droplets are deemed as “positive” if the fluorescence of the droplet is higher than the defined threshold and “negative” if it is lower. Considering the possibility that a droplet can be either positive or negative in each of the three detection channels, there are $2^3 = 8$ possible ways to classify the droplets. Furthermore, the possibility to visualise the data in 1, 2 or 3-D fluorescence dot plots (Figures 6, 7 and 8, respectively) allows to accurately analyse the data and interpret it.

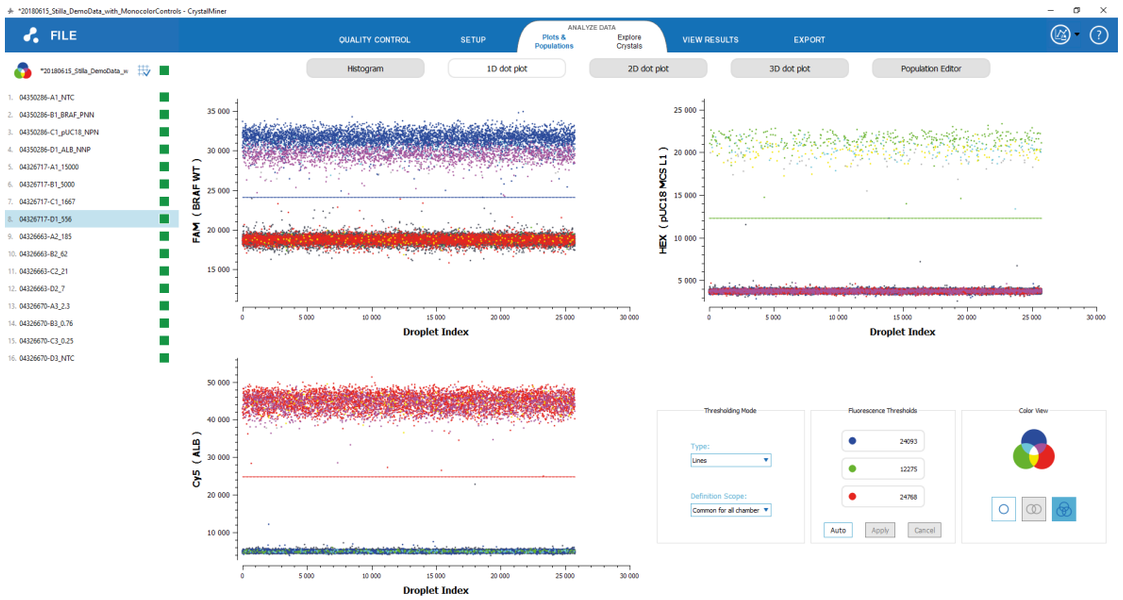


Figure 6: A screenshot of the Crystal Miner software illustrating 1D dot plots in 8-colour mode.

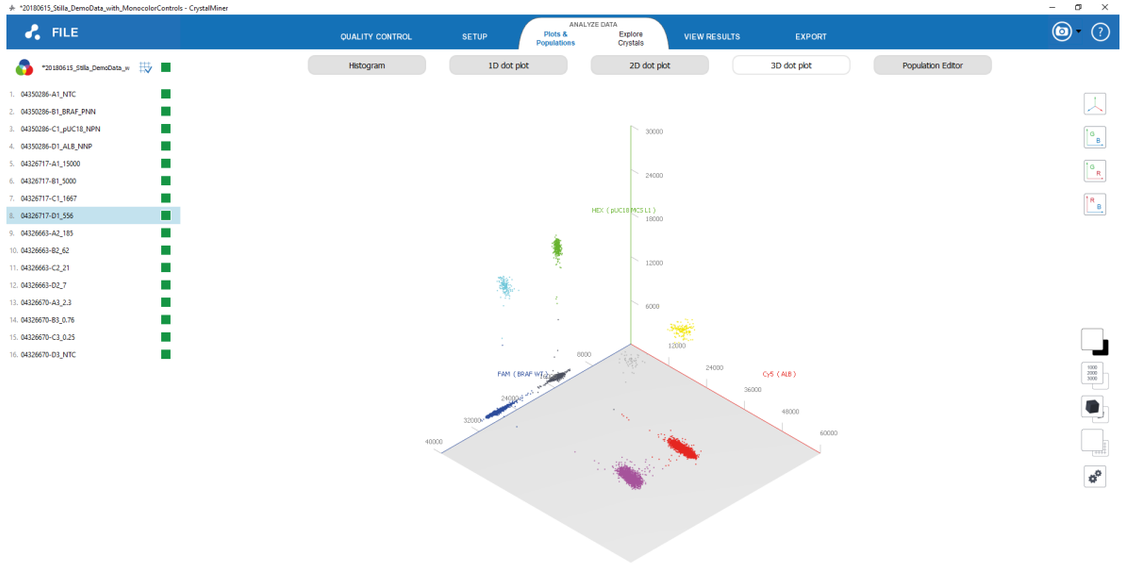


Figure 7: A screenshot of the Crystal Miner software illustrating 2D dot plots in the 8-colour mode.

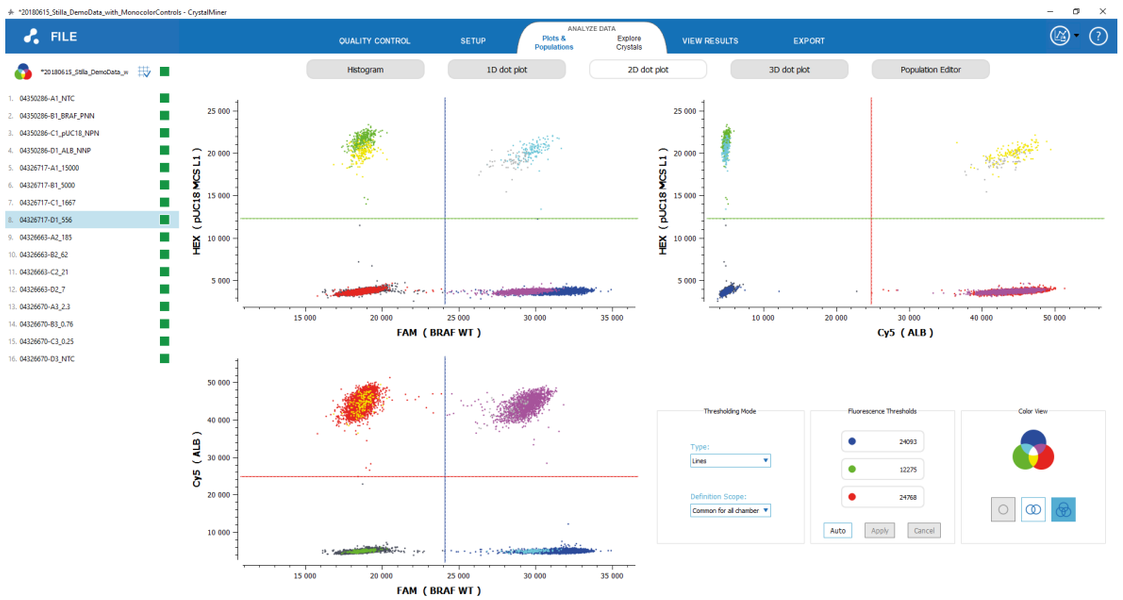


Figure 8: A screenshot of the Crystal Miner software illustrating a 3D dot plot in the 8-colour mode.

Another feature that confers this software reliable is its “white-box” nature with explorable crystal images. This powerful feature aids the user to visualise droplets and confirm that the fluorescence values of the droplets have a homogenous spatial distribution in the crystal. The visualisation in different detection channels aids the users to select the droplet populations of interest and analyse them correctly. The software has a range of quality controls in place to ensure high quality and accurate data reporting. For example, quality flags are a feature linked to the white-box nature of Crystal Miner. The quality flags associated with each well are indicative of the quality control at the well level. Ranging from high to moderate and low, they are an instant indication of the quality of the experiment.

Besides, when multiplexing with several hydrolysis probes, it is sometimes possible that a signal from one fluorophore is detected in more than one channel due to spectral overlap, causing a fluorescence spillover or crosstalk. This effect can be seen in the dot plots and may disturb the automatic threshold computation. Using Crystal Miner, fluorescence spillover can be compensated automatically based on mono-colour controls, easing the thresholds setting. To learn more about spill over compensation see here: https://www.gene-pi.com/item/spill-over-2/.

After compensation (when needed), users can adjust the automatically set thresholds by sliding the corresponding line on the dot plots for the different detection channels, enabling Crystal Miner to distinguish between positive and negative droplets. When a positive population of droplets cannot be separated from the negative population by a line boundary in a 1D dot plot, the Crystal Miner allows the user to define populations by drawing custom zones such as polygons in 2D dot plots. This flexibility to customize populations is another advanced feature of the Crystal Miner software allowing defined populations for specific experimental requirements such as for a drop-off assay, assays based on CRISPR gene-editing, or in cases where multiple targets are colocalised in a single detection channel. Apart from

the three default populations, users can choose to define custom populations using the “Zoning” and “Population” defining features. Briefly, using the Zoning feature, the user can divide the 3D fluorescence space into zones, either using the 1D Line threshold or the 2D Polygon threshold modes (Figure 9), and the software automatically calculates the usual results for the populations in these zones. An example where the 2D Polygon thresholding can be used for defining populations is a drop-off assay and a detailed explanation of this thresholding mode can be accessed here: https://www.stillatechnologies.com/how-to-quantify-drop-off-digital-pcr-assays-with-crystal-miner/

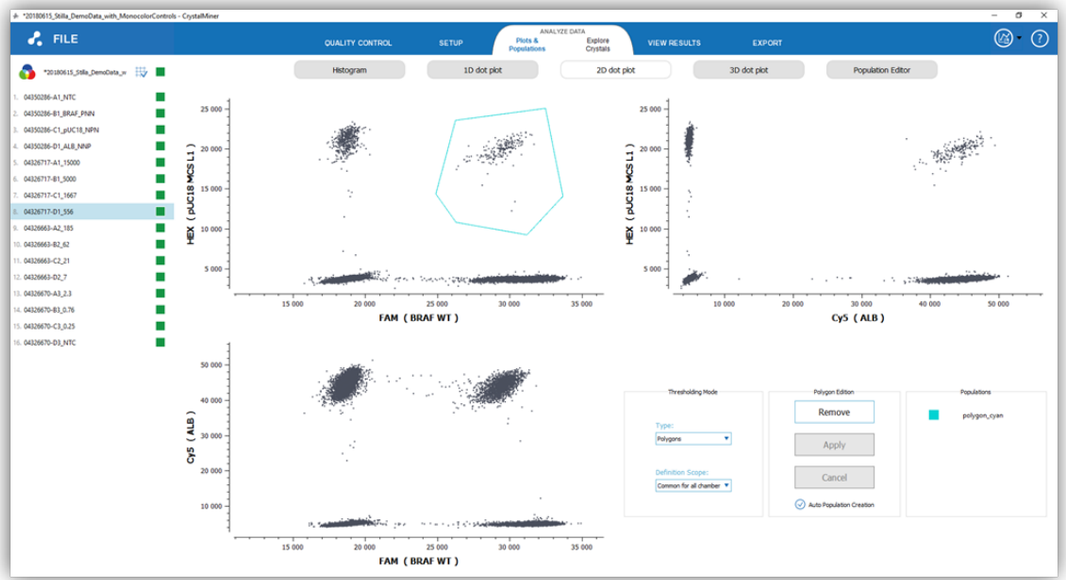


Figure 9. Screenshot of the Crystal Miner illustrating the “Polygon” thresholding mode.

Figure 10. Screenshot of the Crystal Miner illustrating the “population” defining feature.

Further, the “Population” defining feature gives the users the option to manually define complex populations using a building block approach after fluorescence zones have been defined. For example, for a triplex experiment, the user can select or remove the desired populations, customize the colour of the populations and even add specific negative droplets to be excluded from a population in case of drop-off quantification (Figure 10). The results for the additional populations are then displayed in the Results tab of the Crystal Miner software.

Performance indicators to evaluate the amplification efficiency, quantification precision, and detection sensitivity are also provided by Crystal Miner. Some of the quality control features include the separability score, uncertainty curves and the pooling strategy.

The separability score is an indicator for the quality control and for assay optimization. The separability score is proportional to the distance between the positive and negative droplet clusters and is inversely proportional to the sum of Bessel-corrected standard deviation of each cluster. A higher score points to a good amplification efficiency of the target whereas a lower score indicates that the droplets are ambiguous and have intermediate fluorescence values. A detailed explanation of the mathematical calculations for the separability score is provided in the next section.

Irrespective of the separability score and the thresholding mode, each population is associated to a target or a group of targets whose concentration is estimated based on the ratio of positive and negative droplets for the population. As a result, Crystal Miner provides the estimated concentrations together with their confidence intervals at 95% confidence level. The width of this confidence interval is linked to the uncertainty of the measurement and displayed in the form of uncertainty curves.

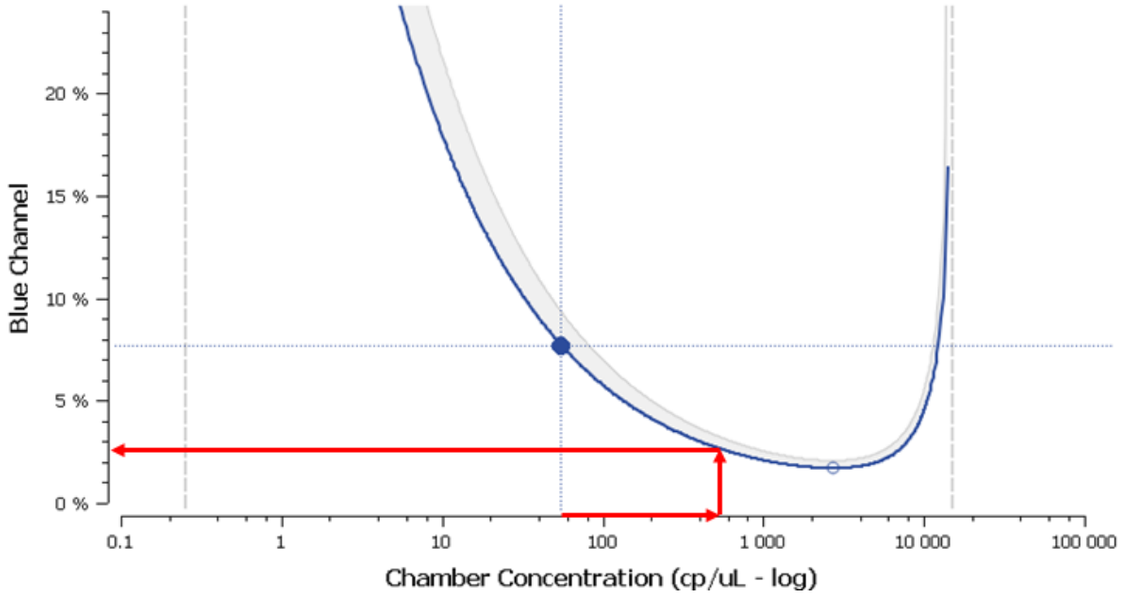


Figure 11: Screenshot of the Crystal Miner illustrating uncertainty curve obtained for a given experiment. In this example, for higher precision, further concentrating the sample would result in a decrease in the uncertainty from 8% to approximately 3%.

Another tool for improving detection sensitivity and quantification precision in case of rare mutations is the possibility to pool wells. Using the pooling feature, users can combine two or more wells containing replicates and increase the analysed volume. The Crystal Miner software automatically calculates the concentration of each target in the pooled wells using the formula based on the sum of positive and negative droplets, briefly:

$$C\left(kpooledwells\right) = -\frac{1}{v} ln\left(1 - \frac{\sum_{i=1}^{k} p_i}{\sum_{i=1}^{k} N_i}\right)$$

w h e r e :

k refers to the number of pooled wells, assuming that these wells are replicates (e.g. from the same sample)

v refers to the droplet volume

N_i refers to the total number of droplets analyzed in well

p_i refers to the number of positive droplets observed in well *i* for the target of interest

An increase in the analysed volume obtained by pooling wells leads to lower sampling and partitioning errors as a consequence of an increase in the total number of droplets analysed, resulting in a lower quantification uncertainty. Assuming the LOB equal to zero, the formula for LOD indicates that the LOD is inversely proportional to the number of pooled wells. Assuming N is the average number of droplets analysed per well, the LOD can be calculated using the formula:

$$LOD\ \left(k\ pooled\ wells\right) = \frac{3}{k\ N\ v}$$

Guidelines for Digital PCR data analysis

Necessary conditions for a digital PCR experiment to follow Poisson Law

A digital PCR experiment can only be exploited under the assumption that the distribution of the target nucleic acid fragments in the generated partitions follow a Poisson Law, which means that:

- the partition volume v is assumed to be constant;
- the encapsulation of target fragments in the partitions is assumed to occur independent of each other;
- the average number of target fragments encapsulated in a droplet is assumed to be constant and equal to C_v, where C is the expected concentration of target fragments in the digital PCR well.

Checking these assumptions is highly recommended to ensure the reliability of the following Poisson formula which estimates the target concentration in the well:

$$C = -\frac{1}{v}\, ln\left(1 - \frac{p}{N}\right),$$

where N is the number of partitions generated in the well and p is the number of observed positive partitions for the target of interest (i.e. those having encapsulated at least one target fragment).

If the generation of the partitions is performed sequentially through time or in parallel in space, the Poisson Law assumption requires that the fluorescence values of the partition are randomly distributed in time or space, and that the distribution of positive partitions for the target of interest is also random in time or space.

The random distribution assumption can, for example, be compromised in case of fragment sedimentation in the loaded sample, or in case of non-homogeneous temperature heating during the PCR.

The white-box Crystal Miner software allows users to visualise all images of the droplet crystals and to highlight potential droplets associated with rain or unexpected clusters in the dot plot graphs. Such functionalities enable the user to perform quality control for partition volume assumption as well as the random distribution assumption.

Estimation of the theoretical and experimental sources of digital PCR measurement errors

There are two categories of digital PCR measurement errors:

- The theoretical Poisson error $\varepsilon_{Poisson}$ which cannot be avoided and accounts for both the sampling error and the partitioning error.
- All the experimental sources of digital PCR measurement errors, which should be mitigated as much as possible. They include the following sources:
  - human manipulation errors (e.g. pipetting errors);
  - biological errors (e.g. target competition, non-specificity, non-optimal amplification efficiency);
  - instrument-related errors (e.g. thermal cycler, fluorescence reader);
  - software-related errors (e.g. partition detection, partition classification by fluorescence thresholding);
  - human interpretation errors (e.g. manual adjustment of data analysis).

The formula for the Poisson error at 95% confidence level is the following:

$$\varepsilon_{Poisson}(0{,}95) = \frac{C_{max}(0{,}95) - C_{min}(0{,}95)}{2\,C}$$

where, using the notations defined in the previous section:

$$C = -\frac{1}{v}\,ln\left(1 - \frac{p}{N}\right)$$

$$C_{min}(0,95) = -\frac{1}{v}\ln\left[1 - \left[\frac{p}{N} - 1.96\sqrt{\frac{\frac{p}{N}\left(1-\frac{p}{N}\right)}{N}}\right]\right]$$

$$C_{max}(0,95) = -\frac{1}{v}\ln\left[1 - \left[\frac{p}{N} + 1.96\sqrt{\frac{\frac{p}{N}\left(1-\frac{p}{N}\right)}{N}}\right]\right]$$

The total variability of digital PCR measurements can be evaluated by performing a sufficiently large number of pure digital PCR replicates (e.g. by loading the same sample in at least digital PCR wells), by collecting the target concentration values measured by the digital PCR system, and by calculating their coefficient of variation corrected with Bessel's correction:

$$c_v = \frac{\sigma}{\mu}\sqrt{\frac{n}{n-1}}$$

Finally, the experimental relative uncertainty at 95% confidence level can be deduced by subtracting the theoretical Poisson error from the measured total variability:

$$\varepsilon_{Experimental}\left(0,95\right) = 1.96\ c_v - \ \varepsilon_{Poisson}(0,95)$$

Optimization of multiplex digital PCR assays from data processing

Optimization of multiplex digital PCR assays requires maximizing the amplification efficiency of each target of the assay while avoiding target competition and non-specificity issues. If targets are involved in the multiplex assay, it is recommended to first optimize a simplex experiment for each target, then optimize the possible duplex experiments, triplex experiments, and so forth until the wished -plex experiment.

The optimization parameters include the choice and the concentration of the primers and probes for each target, as well as the PCR conditions (number of PCR cycles, the duration and temperature for the denaturation step, the annealing step and the elongation step).

One relevant and objective criterion to quantify the amplification efficiency for a target of interest is the separability score between two clusters of points:

- the "positive" cluster of points representing the fluorescence of the positive partitions (i.e. the partitions which have encapsulated at least one target fragment);
- the "negative" cluster of points representing the fluorescence of the negative partitions (i.e. the partitions which did not encapsulate any target fragment).

The formula of the separability score for the target of interest is the following:

$$separability = \frac{\mu_{POS} - \mu_{NEG}}{\sqrt{\frac{p}{p-1}}\ \sigma_{POS} + \sqrt{\frac{q}{q-1}}\ \sigma_{Neg}}$$

where:

p is the number of positive partitions observed for the target of interest;

q is the number of negative partitions observed for the target of interest;

μ_{POS} is the mean fluorescence value of the positive partitions;

μ_{NEG} is the mean fluorescence value of the negative partitions;

σ_{POS} is the standard deviation of the fluorescence values of the positive partitions;

σ_{NEG} is the standard deviation of the fluorescence values of the negative partitions.

In order to optimize a k-plex experiment, the objective is to maximize the separability score for each of the k targets.

Finally, the recommended optimization process is as follows:

- Modify only one parameter at a time;
- In order to determine if the new conditions are better than the current ones, perform at least pure replicates of the current conditions and at least n=30 pure replicates of the new conditions, then apply for each of the n targets then the Mann–Whitney U test to the separability scores obtained for the targets using the current conditions versus the new conditions (using one-tailed hypothesis and 0.05 significance level);
- If the Mann–Whitney U tests indicate that the new conditions lead to equivalent or statistically higher separability scores for all the targets, then the new conditions do not cause regression and can be kept for a further iteration.
- A separability score of 4 is the lowest acceptable value for any target of interest. We recommend further assay optimization when scores lower than 4 are obtained in order to ensure confident fluorescence threshold placement.

Evaluating the linearity performance of a digital PCR assay:

In order to assess the linearity performance of a digital PCR assay with respect to a given target, it is recommended to perform an m-point dilution range of the target (ideally with m>5) along the whole dynamic range of detection specified for the digital PCR system, and to include a negative control point for quality control. Besides, each dilution point should be performed in at least n=3 replicates to ensure a robust evaluation of the linearity.

For example, the Naica System offers a dynamic range of detection from 0.2 cp/µL to 20 000 cp/µlin the digital PCR well, so a suitable linearity study could include the target concentrations 20 000, 2 000, 200, 2, 0.2 and 0 cp/µl.

The standard linear regression, based on the Ordinary Least Squares (OLS) method, assumes homoscedasticity, i.e. the assumption that the variance of the errors (or residues of the regression) is constant across all points in the dilution range. However, this assumption is false for digital PCR, because the variance of the errors is much larger at very low concentrations and at very high concentrations. This non-homogeneity of the variance is called heteroskedasticity.

The recommended methodology consists in applying a robust Weighted Least Squares method (WLS method), which considers as weight for each dilution point the inverse of the variance of the replicates at this dilution point. The higher the coefficient of determination obtained for the regression, the better the linearity. However, the most important criterion is not the value but the fact that there is a straight line which passes close enough to each point of the dilution range, with a slope as close as possible to the value , and ideally, a bias as close as possible to the value .

For more details, please see https://www.stillatechnologies.com/detection-of-her2-copy-number-variation-with-crystal-digital-tm-pcr/. An online calculator to determine CNV from a digital PCR experiment is also available here: https://www.gene-pi.com/statistical-tools/cnv/.

Conclusion

In a nutshell, Crystal Digital PCR technology can be implemented and optimized for accurate and reliable detection of genetic alterations in a broad range of nucleic acid quantification assays. Additionally, the Crystal Miner software, equipped with advanced quality control tools, enables users to robustly identify target mutations in samples of varying origins. Crystal Digital PCR, coupled with the Crystal Miner software, is a high precision nucleic acid quantification tool that can aid researchers working in various nucleic acid testing fields to explore new applications.

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Chapter 19

Setup and Analysis of qPCR Transcript Assay Arrays

Ensuring MIQE Compliance

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Section 1

Summary

Utilising focused miRNA or mRNA qPCR plate arrays is an excellent way to find transcripts that are regulated in an experimental context. However, setting up and running these screening experiments is not the same as performing a standard qPCR experiment. Experimental design and execution require compromises that immediately preclude the implementation of the full set of recommendations as outlined in the MIQE guidelines {Bustin, et al., 2009}. This chapter deals with how best to set up qPCR-focused array screening experiments, discusses the compromises to MIQE in a screening experiment and emphasises the importance of post-screen validation of screening results to ensure the data are valid and comply with the principal MIQE recommendations.

Section 2

Introduction

qPCR plate arrays are an excellent way of finding new transcripts within a pathway, small set of pathways or a selected collection of gene targets. Messenger RNA qPCR assay arrays have been around at least since 1998 when S.A. Biosciences (now part of Qiagen) first made them commercially available. Today, both mRNA and miRNA qPCR assay plate arrays are available from many commercial vendors in both 96- and 384-well plate formats. In many cases, companion reagents are also available specifically designed for their cognate arrays. These reagent kits offer guidance to the researcher on how best to setup the RT and PCRs using their arrays. An investigator comfortable performing standard qPCR experiments with many samples and few assays will find that running qPCR arrays requires a different approach. The primary difference is that each plate contains multiple assay targets and is dedicated to a single or only a few samples. This results in the need to run many plates to encompass all the samples in the experiment. Data analysis has to be approached differently as well, as it is not practical to set up a screening experiment and be 100% MIQE compliant. The information in this chapter will discuss the important criteria for performing a screening experiment utilising arrays and considers the rationale and procedure for performing a post-screen validation experiment to obtain full MIQE compliance for the candidate targets.

This chapter focuses on qPCR assay arrays designed to interrogate the transcriptome. It is theoretically possible to run SNP and other DNA-based assay arrays using qPCR on multiple samples. The same basic principles and procedures outlined in this chapter, without the reverse transcription (RT) steps, would be applicable to those array types as well.

Section 3

What about MIQE ?

As stated above, it is not possible to run a 100% compliant qPCR multi-assay screen. What does this mean? The main points are contrasted in Table 1. The first main difference is the lack of technical replicates. Although it is possible to run the same sample multiple times on multiple array plates, it is not financially practical. It is much better to run multiple biological replicates per biological group instead, so accounting for the biological diversity within the group. However, there will be fewer of these than in a standard RT-qPCR experiment. The two main controls for an RT-qPCR experiment are not present, the no-template control to gauge possible template contamination and the no reverse transcriptase control to measure any DNA contamination. It is just not practical to include these controls within a qPCR screen. In a standard RT-qPCR experiment, one or more (preferably) validated transcripts (Vandesompele et al., 2002) should be used to normalise the data set for loading. The one advantage of a screen that interrogates the levels of many transcripts is that a ‘global normalisation’ protocol can be used analogous to what is used with microarrays (Mestdagh et al., 2009), (D'haene et al., 2012). The advantage of this approach is that the data set can be normalised without previously validating reference genes. It also offers an opportunity to validate reference genes included within the assay set on the plate that can be used in a post-screen validation of candidate assays. Finally, since there are so many different transcript qPCR assays within an assay screen, it is not possible to determine PCR efficiency and the limit of quantification (LOQ) for each assay.

Therefore, the cut-off for valid data has to be chosen without numerical data and thus, some data may in fact not be valid for analysis. Lastly, the investigator has to take it on faith that the assays within the array have high template specificity. Taking all of these issues together, it becomes clear that a post-screen validation experiment is essential to make sure the candidate transcripts found in the screen are still valid for a larger sample set for each biological group using a set of experimental conditions where the MIQE Guidelines can be fully applied.

Standard versus Screening - Experimental Differences		
Item	Standard qPCR	qPCR Screening
Technical Replicates	2-3	None
Biological Replicates	10s - 100s	Small Subset
RNA Quality	Best Possible	Best Possible
No-RT Controls	Yes	No
No-Template Controls	Yes	No
No-assay Controls	No	Yes
LOQ - Assay	Determined	Unknown
PCR Efficiency - Assay	Determined	Unknown
Assay Specificity	Determined	From Supplier
Inter-plate Calibrators	Not usually	Yes
Assays/Plate	Single to a Few	10s - 100s
Data Normalization	Individual Transcripts	Global Mean

Table 11.1 - Standard versus Screening - Experimental differences

Planning a RT-qPCR Screening Experiment

1. Samples and RNA Preparation
2. qPCR Plate Arrays and Reagents
3. Equipment
4. RT

As with any qPCR experiment, the entire workflow should be thought through completely to ensure an experiment has a good chance of generating a credible result, at least from a technical point of view. In a standard qPCR experiment, the transcript assays being tested have already been selected using other methods such as next-generation sequencing (NGS), microarray analysis or have been derived from previous experimental data or from the literature. For a standard qPCR experiment, the number of samples required to achieve statistical significance for any two biological groups is determined using a power analysis on a subset of samples for each target assay being investigated (Kloprogge et al., 2014). In a screen this approach is not practical as the aim is to find novel transcript targets for future study. In a screen, a minimum of seven samples per biological group is optimal to get complete statistical analysis of all the essential parameters of the screen. More samples can be used and will add power for low abundance transcripts, but at a higher sample and materials cost. The number of samples to use depends upon the starting material. For experiments utilising isogenic tissue culture cells will require fewer numbers. In this case, a true biological replicate is not really possible and best practice would use replicates from experiments done at different times that will add to the validity of the results. For inbred laboratory animals, the number tested should be higher than for tissue culture cells but can be lower than for a wild type population. Human samples may require as many as 20 samples per biological group to compensate for natural

variation (see the Summary below). The only practical way to achieve such patient numbers is with a post-run validation experiment. In the end, the number of samples to be tested becomes an issue of time, equipment and labour resources, the configuration of plate pack sizes by manufacturers and the experimental budget. Although a post-run validation experiment should be performed regardless of the sample source for the data to be fully MIQE compliant, validation of the final results will be more certain using the largest practical sample size for the screen. Investigators familiar with microarray experiments will want to screen their entire sample set. For a qPCR screen the strategy should be to screen enough samples to get as much information as possible but then use the post-screen validation to run the complete sample set. This is the most economical and most MIQE compliant approach. A discussion of post-run validation experiments will be found later in this chapter.

Samples and RNA Preparation

A major consideration is the identification and treatment of the samples themselves. At this stage, there is no difference between a qPCR screening and a standard qPCR experiment. Care should be taken to make sure that each sample comes from a subject that has been treated correctly for its biological group and that each member of a group belongs to that biological group.

In my experience of running a full service qPCR core facility for over 13 years, I found that investigators were very careful how they treated collected tissue samples from the -80°C freezer through RNA isolation. However, they were not as careful in how the tissue was initially handled from the collection site until put into freezer storage. The first moments from dissection until the sample is properly stored are the most critical. Once a sample has suffered RNA degradation, downstream compensation is not possible. Thus, a preplanned workflow for all samples must be worked out in advanced and strictly adhered to during sample collection. In one extreme case, an investigator found that samples excised from a rat had to be homogenised immediately following dissection to ensure high quality RNA.

Isolation of RNA from plants, fungi and microbes involve different, but no less problematic, approaches but the basic philosophy is the same as for sample collection and stabilisation of RNA from animals. Again, it is critical that the RNA is stabilised as soon as possible and any degradation avoided at all costs.

Methods and reagents for the isolation of high quality total RNA are available from a multitude of companies. Whatever the method of choice, it is critical to use the same method for all the samples used in a study, both for the screen itself and any follow-on validation experiments. For the isolation of miRNA, there are vendor recommended steps for optimising miRNA isolation and

these recommendations should be followed to the letter. The mantra of the screen is uniformity so that biological differences will have the best chance of being detected above any technical noise generated in the multiple steps involved in performing the screen.

RNA quality is a subject that is much talked about in terms of qPCR in general but I'm afraid is also much ignored. For any qPCR experiment to be successful, it is critical that the quality of the RNA in each sample be similar. You will note that I did not say of the highest quality, comments above not withstanding. Some samples will never be of high quality, those from FFPE samples for example. However, it is still critical that those samples have similar RNA quality. There is good evidence that having the highest quality RNA possible is essential for the best outcome in a RT-qPCR experiment (Vermeulen et al., 2011), (Becker et al., 2010). For purified RNA, the Agilent 2100 Bioanalyzer or Bio-Rad Experion chip-based capillary electrophoresis systems are best for assessing RNA quality while for miRNA, the 2100 works best. Examples of what high quality RNA looks like when assayed on an Agilent 2100 can been seen in Figure 11.1. Figure 1A shows high quality total RNA without miRNA while Figure 1B shows the extra peak (red arrow) when small, non-coding RNAs have been isolated as well.

Figure 1 - Agilent 2100 Analysis of Total RNA

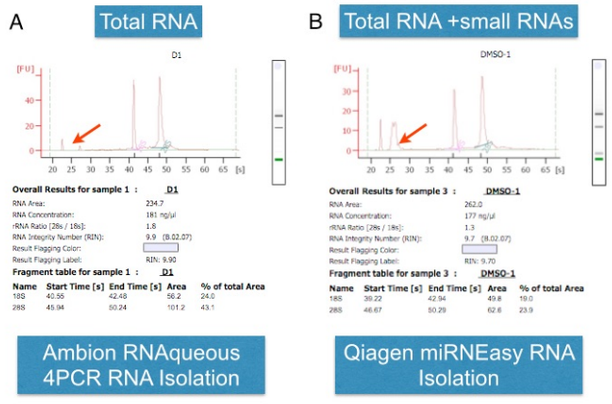


Figure 11.1 - Agilent 2100 Bioanalyser analysis of two total RNA preparations. A- Total RNA preparation using the Ambion RNAqueous 4PCR kit showing a lack of small non-coding RNAs (arrow), B- Total RNA prepared using a Qiagen miRNEasy isolation kit showing a peak representing small non-coding RNAs (arrow).

There is an alternative to RNA purification and that is whole-cell lysates. A special reagent is used to lyse cells and stabilise the RNA long enough for cDNA to be made in a RT reaction. Kits from multiple suppliers are available for this purpose. However, the crude lysates have a finite life span, even at -80˚C. An alternative is the RNA prep method from ZyGem that involves washing the cells in PBS in the tissue culture plate, adding buffer bearing a thermostable hyperactive protease and digestion of whole cells at 75˚C on a raised heater block in situ for 5 minutes. The main advantage of this method is there are no proteins left, in particular nucleases or DNA binding proteins on the genomic DNA (gDNA). Thus the gDNA can provide valuable information about how many cells were lysed and all the RNA species are present without loss. For RNA quality measures with whole cell lysates, the 3'-5' method is recommended for mRNA (Nolan et al., 2006a), (Vermeulen et al., 2011). For miRNA, running a miRNA found in most tissues or small ncRNA qPCR assay is best to ensure the presence of small ncRNAs in the preparation and to compare across samples for similar amplification profiles and quantification cycle (Cq) values. This method can be used for isolated RNA preparations as well. An example using the ncRNA SNORD 48 qPCR assay utilising total RNA from FFPE samples is shown in Figure 11.2. The Nanodrop data in Figure 11.2 show the sample concentrations and that they are of similar purity from the A260/280 measure, although the A260/230 measures were not all ideal. The similar shape of the amplification curves rules out RT and/or PCR inhibition, the similar Cq values validate the Nanodrop values for RNA concentration. Figure 3 illustrates a ZyGem-prepared whole cell lysate dilution series with two Exiqon human miRNA individual assays. Up to 8 µl of whole cell lysate, when prepared in this fashion, can be used per Exiqon RT reaction with excellent results. Using whole cell lysates from other vendors at this volume inhibits the Exiqon RT reaction. The variable volume test confirms there was no RT or PCR inhibition in whole cell lysates made in the manner.

Figure 2 - Nanodrop & SNORD 48 ncRNA Assay Data

Nanodrop Data

Sample	ng/µl	260/280	260/230	Mean Cq
1	164	2.02	1.87	24.19
2	162	2.09	2.09	23.98
3	127	1.98	1.6	24.32
4	189	2.04	0.78	25.84
5	162	1.95	1.64	25.03
6	189	2.03	1.23	23.89
7	241	1.99	1.75	24.11
8	244	1.99	1.95	24.24
9	167	2	1.45	24.95
10	260	1.99	0.86	25.96
11	178	1.96	0.7	24.95
12	170	1.99	0.81	24.96

qPCR - Duplicate Curves

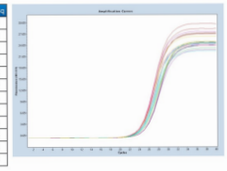
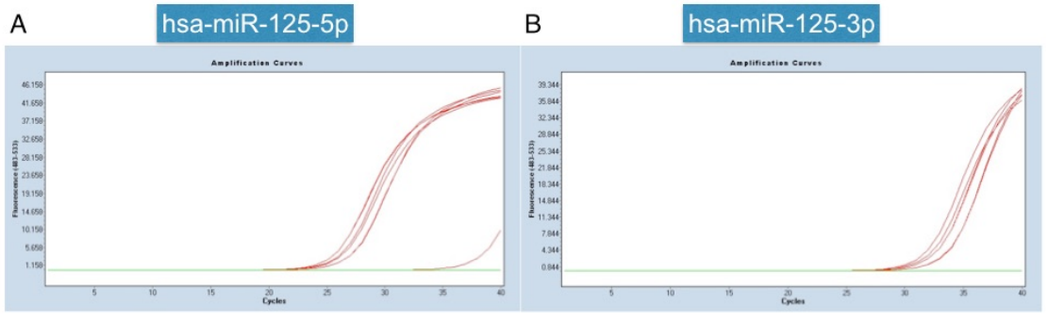


Figure 11.2- Utilising the SNORD 48 ncRNA assay and the Nanodrop spectrophotometer to access RNA quantity and quality in total RNA from FFPE samples. Total RNA from human FFPE samples was made using a Qiagen RNeasy FFPE kit. RNA quantity and purity was determined using a Nanodrop. RNA concentrations were more than sufficient for further analysis and A260/280 ratios were near ideal. Some of the A260/230 ratios were less than ideal prompting an RT-qPCR test. Potential RT or qPCR inhibition and presence of small non-coding RNAs were tested using an Exiqon SNORD 48 individual assay. Samples were run in duplicate. All amplification curves had a similar shape and Cq values were sufficiently close showing no RT or PCR inhibition and that the sample concentrations were within 2-fold validating the Nanodrop data.

Figure 3 - RT or PCR Inhibition Testing by Varying Template Volume



Lysate Volume	125_5p (Cq)	125_3p (Cq)
2	26.87	33.47
2	26.93	33.44
4	25.95	32.31
4	26.05	32.26
8	25.35	31.55
8	25.35	31.86
NTC		

Figure 11.3 - RT or PCR inhibition testing by varying template volume in the RT reaction. Two individual Exiqon human miRNA assays were used to determine what volume from a ZyGem whole cell lysate preparation could be used in an Exiqon RT reaction without inhibiting the assay. This experiment also shows that there was no RT or PCR inhibition in the whole cell lysate preparation due to the linearity in the regression curves for the 3 volumes tested. A- hsa-miR-125_5p assay; B- hsa-miR-125_3p assay.

Finally, the topic of inhibition of the reverse transcriptase and/or amplification steps should be addressed. For reverse transcriptase inhibition, an RNA spike-in assay for a target that is not present in the RNA preparation is used. Following RT of an aliquot from all samples and one containing the spike-in with H2O instead of RNA are run in a qPCR reaction. The Cq values of each sample should be very similar to one-another and to the H2O control. If there is variability within the sample set, those with the highest inhibition should be removed from the sample set for each biological group. The Exiqon RT step includes a small RNA spike-in but otherwise, potential inhibition of the RT step has to be performed using reagents provided by the investigator. Similarly, running the SPUD assay (Nolan et al., 2006b) will determine whether there is PCR inhibition in any of the samples, as long as they are not from potatoes. An analogous assay from an animal or microbial source should be used for such samples. Since neither type of inhibition will be uniform within each sample set, RT or PCR inhibition can add technical error to any RT-qPCR experiment. Due to the cost of running a qPCR screen, it is critical to make sure that reaction inhibition is not an issue for the samples prior to initiating the screen.

qPCR Plate Arrays and Reagents

As mentioned above, there are many choices in the type and source of mRNA and miRNA arrays available. The choice of system will depend on the questions being asked and what instrumentation is available. The most important distinction is between 96-well and 384-well instruments, with the latter having the advantage that more assays and/or samples can be interrogated using a 384-well plate array. Furthermore, experimental needs will determine the choice of assay array. That is, if targets are already narrowed down to a small finite set, multiple arrays can be prepared on each plate, which will allow more than one sample to be run per plate. Conversely, if the aim is to identify candidate transcripts, then it will be necessary to run the largest possible array available with only a single sample per plate. It is imperative to think about this carefully before ordering array plates to ensure setup and needs are properly matched. If only a finite number of targets are being analysed for each array, it is important to know which transcripts to use for data normalisation and make sure those are present in the array set. It is inadvisable to depend upon the vendor's choice as none of the assays the vendor uses for every array may turn out to be appropriate for individual experimental conditions. This can only be determined by using software and the Cq values from the finished array. The use of a full plate array allows the application of global normalisation to the data set and help find suitable transcripts for a follow-on validation experiment, as described below.

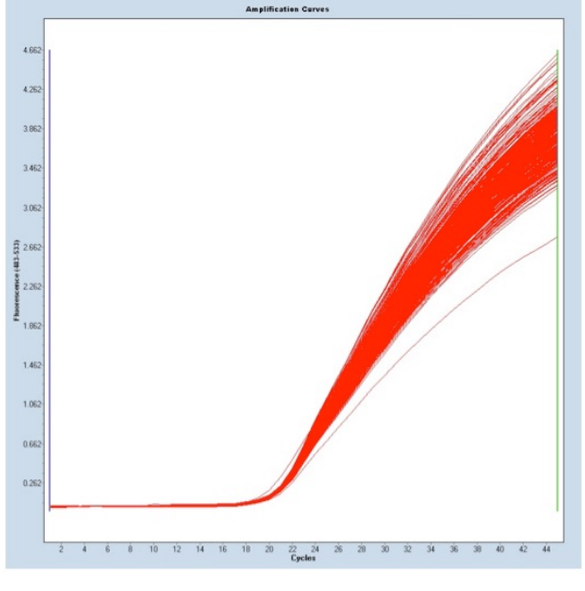
As a core laboratory, we were well known for making our own RT and qPCR master mixes. However, qPCR arrays are probably best run using the recommended reagents supplied by individual vendors, as they have been optimised to work with the array assays. Using reagents from other vendors will probably lead to less than optimal results. Further, some vendor reagents (eg., Exiqon) contain control elements that will be useful in downstream data analysis. In the same vein, it is critical to follow the vendor's instructions for preparing and running the arrays to the letter.

Equipment

It should go without saying that all equipment used in a screen should be working optimally. This is especially true of pipettes. If the lab's single and multi-channel micropipettes have not been calibrated within the last 6 months they should be calibrated by a reputable company. If the experimental design includes the use of a liquid handling robot, it is important to ensure that it is working correctly. The term accuracy and precision is used a lot with robots but it is important for hand pipettes as well. Accuracy pertains to the consistency of how much volume is put into replicate wells. This is especially critical when single aspiration/

multiple dispense steps are present within the program but applies to hand pipetting as well. Clearly, the data will not be consistent if individual instruments are not giving excellent accuracy. Precision refers to the volume being dispensed. A well-calibrated hand micropipette will be very precise. However, it is common for liquid handling robots to not dispense the exact volume written in the software. Running the reaction with too little or too much master mix is not ideal and may affect array performance. The main issue is that there is too much or too little (usually the case) to fill the entire plate in the source plate, even though the researcher has calculated how much PCR reagent will be required. It is pretty irritating to find this out during the actual experiment. It is useful practice to pre-run the robot program to ensure acceptable precision before starting the actual array experiment. If the accuracy and/or precision of the instrument are not acceptable, it is advisable to check with the vendor on how to best solve the problem. An example of an accuracy test for an entire 384-well plate using single aspiration and quadruplicate dispense steps from an 8-channel tool on a Beckman Biomek 2000 is shown in Figure 11.4. Being able to generate this kind of tight control data using either automation or from utilising a hand multichannel pipette is essential to a successful screen. Similarly, precision can be checked by aspirating random wells with a well calibrated hand pipette set to the desired dispense volume.

Figure 4 - Biomek 2000 Pipetting & LC480 qPCR Instrument Test



384-wells - 10 µl Rxns

Mean Cq	StDev	%CV
20.62	0.07	0.33

Figure 11.4 - Biomek 2000 program quality control test. A single complete master mix bearing a diluted oligonucleotide template and primer/probe for a human β-Actin qPCR assay were pipetted by a Biomek 2000. Using the same 8-tips on an 8-channel tool to fill the entire plate, 10 µl of complete qPCR master mix were added to each well using single aspiration and quadruplicate dispenses in a loop until the entire 384-well LC480 plate was filled. The amplification curves following analysis on the LC480 instrument for 45 cycles are shown. The table shows tight clustering for the Cq values from all wells.

Most investigators assume that their qPCR instrument is working properly. This may be the case if the instrument belongs to a single lab and it is maintained under a service contract. However, if an instrument is shared with another lab or if it has not been properly cared for, it would be best to test the instrument block for contamination, detection sensitivity and uniformity.

This is done by preparing a complete master mix using a template concentration that will stress the system with a relatively high Cq value, say 30-32 cycles, together with the volume/well intended for the screen and cycling conditions as close as possible to the ones used by the array plates. When the array is finished, Cq values are imported into a spreadsheet such as Microsoft Excel and the data are oriented to correspond to the array on the plate. This is easily achieved using “Paste Special” and the “Transpose” option to change data in rows to columns or visa versa. When the Cq values are properly arrayed in either a 96- or 384-well pattern, the means (avge) and standard deviations (stdev) are calculated at the end of each row and bottom of each column. This allows a calculation of the % correlation of variation (%CV) by dividing the standard deviation by the corresponding mean and multiplying that value by 100. The %CV values for all rows and columns should be nearly the same. If they vary much, mean values need to be analysed to detect possible outlier wells or worse yet, a pattern such as outer wells verses the inner wells.

If the instrument doesn’t pass this test, the vendor needs to be contacted on how best to rectify the issue(s) prior to running an array. If the qPCR instrument is shared, it is important to make sure to set aside a period of time for running the entire set of array plates so that the time from when a plate is prepared to when it is run is as consistent as possible. It is also good practice not to stretch out this procedure over more days than necessary. The plates should all be run as soon as possible from when the first array plate is setup.

RT

In keeping with the theme of uniformity, it is critical that all samples be reverse transcribed at the same time, using the same reagents and the same procedure following the manufacturers recommend protocol to the letter. As an example, I will use an Exiqon miRNA screen and validation that was performed for Dr. Justin Yustein at Texas Children’s Hospital, part of the Baylor College of Medicine, in the Texas Medical Center in Houston, Texas. The samples came from a genetically engineered mouse model for osteosarcoma. The control samples and bone tumours (Figure 11.5) came from the femur of the mouse. RNA was prepared from samples frozen immediately following excision from the mouse using the Exiqon miRCURY RNA isolation kit. RNA quality was determined by Agilent 2100 Bioanalyzer analysis as can be seen in Figure 11.6. Each sample had a similar total RNA quality. RNA concentrations and A260/A280 ratios were

determined using a Nanodrop. A combined table of all of this information was assembled as shown in Table 11.2 and is recommended for any screening experiment.

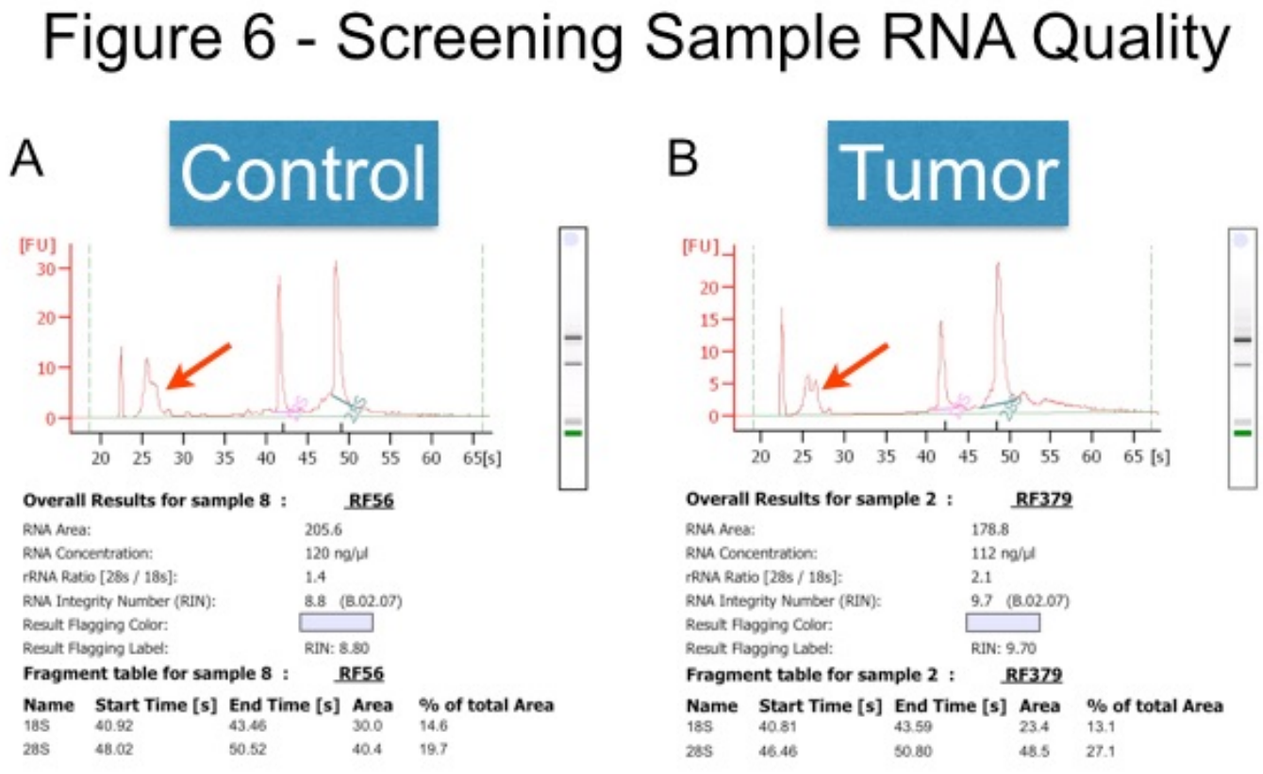
RNA Sample Data							
Sample ID	Sample Type	RNA (ng/μl)	A260/ A280	2100 RNA (ng/μl)	2100 RIN	miRNA peak	Vol (μl) approx
T-1	Tumor	155	2,20	112	9,7	Yes	5,0
T-2	Tumor	260	2,10	263	10,0	Yes	5,0
T-3	Tumor	220	1,98	120	10,0	Yes	5,0
T-4	Tumor	160	2,00	139	9,8	Yes	5,0
C-1	Control	120	1,96	139	9,3	Yes	5,0
C-2	Control	120	2,06	120	8,8	Yes	5,0
C-3	Control	130	2,10	162	10,0	Yes	5,0
C-4	Control	240	2,08	300	10,0	Yes	5,0

Table 11.2 - RNA Sample Data

Figure 5 - Mouse Bone Tumor

Figure 11.5 - Mouse femur bone tumor. A dissected mouse with a distended tumor (osteosarcoma) in the femur is shown. Total RNA was isolated from tumor and cognate control animals for miRNA screening.

Figure 11.6 - Agilent 2100 Bioanalyzer analysis of representative total RNA samples used in the miRNA screen. A- Control sample showing RNA quality (RIN) and the presence of small ncRNAs (arrow); B- Tumor sample showing RNA quality (RIN) and the presence of small ncRNAs (arrow).



Section 5

Performing a qPCR Screen

1. Sample Preparation, cDNA Synthesis and qPCR Strategy
2. miRNA Array Sample Setup, Dispensing and qPCR
3. Data Analysis
4. Data processing
5. Statistics

It should go without saying that before initiating the screen, all materials should be at hand and all equipment available and ready for use. The last thing one wants to happen is to have a delay of any kind once the assembly of assay plates has begun. In this example, there was only a single miRNA array plate available from Exiqon for mouse/rat samples. There are now two 384-well plates for mouse/rat and for human. This doubles the number of plates needing to be run and the logistics needed to do so. When there is more than one plate for each sample being processed, it is critical that they are setup and run back-to-back on the qPCR instrument utilising a 2-fold large PCR master mix containing the sample cDNA to ensure data uniformity for each sample on the two arrays.

Sample Preparation, cDNA Synthesis and qPCR Strategy

The first step is to dilute each RNA sample to the same concentration using nuclease-free H2O as shown in Table 11.3. The final sample concentration will be dependent on the manufacturers requirements. It is best to do this just before starting the screen, keeping the RNA concentration high as long as possible. We used DNA low-bind tubes from Eppendorf to minimise RNA binding to the tube at the relatively low final concentration of 5.5 ng/µl as recommended by Exiqon. The protocol for cDNA synthesis can be seen in Table 11.4. Following cDNA synthesis

for all eight samples, the first two samples were prepared and the miRNA array plates run on the first day. Note that one control and one tumour sample were run on day 1. On day 2, the remaining six samples (six plates) were setup and run. To keep the samples balanced for each day, three controls and three tumour samples were processed on day 2. Only two screening arrays are run on day 1 due to the time required for cDNA synthesis and getting ready for PCR plate processing mode. The run order presented above helps prevent any potential bias that could have occurred had we run two controls first day followed by the remaining controls and tumour samples. Depending on the number of biological groups and the number of samples per group, a similar strategy should be implemented for any qPCR screen to maintain balance in the sample run order.

RNA Sample Dilutions						
		(ng/µl)	(µl)	(µl)	(µl)	(ng/µl)
Sample ID	Sample Type	RNA Conc	Nuc-free H2O	RNA Vol	Final Vol	Final RNA Conc
T-1	Tumor	155	48	1,8	50	5,5
T-2	Tumor	260	98	2,1	100	5,5
T-3	Tumor	220	98	2,5	100	5,5
T-4	Tumor	160	48	1,7	50	5,5
C-1	Control	120	48	2,3	50	5,5
C-2	Control	120	48	2,3	50	5,5
C-3	Control	130	48	2,1	50	5,5
C-4	Control	240	98	2,3	100	5,5

Table 11.3 - RNA Sample Dilutions

miRNA cDNA Reactions			
	Samples =	4- Control	4- Tumor
Exiqon RT Reagents			
RT Master Mix		1.0 Vol	8.5 vol
Reagent	Final Amount	Volume (µl)	Volume (µl)
Nuclease-free H2O	*****	9,0	76,5
5X Reaction Buffer	1X	4,0	34,0
RNA spike-in	1X	1,0	8,5
Enzyme Mix	1X	2,0	17,0
	Total (µl)	16,0	136,0

Table 11.4 - miRNA cDNA Reactions

Exiqon RT Protocol

1) For each RNA sample (8) need to make 1-20 µl cDNA reaction = 1 tube/sample

2) Pre-label the tubes

3) Add 16 µl RT Master Mix to each tube = 8

4) Add 4 µl diluted sample RNA (5.5 ng/µl) to 1 PCR tube in a strip

5) Run in the ABI thermocycler as follows:

42˚C - 60 min

95˚C - 5 min

20˚C - soak

6) Store the cDNA tubes at 4˚C until used - do not dilute until day of use

miRNA Array Sample Setup, Dispensing and qPCR

The process for diluting the cDNA samples and setting up the array plates for days 1 and 2 are outlined in Table 5. In this example using Exiqon reagents, the cDNAs are diluted, mixed with an equal volume of 2X master mix and dispensed using a Biomek 2000 liquid handling robot. In this program, one set of eight tips was used to dispense the entire plate. The program has a single aspiration followed by four dispense steps in a repeat loop until the entire plate was filled. It was found that four dispenses per aspiration were the maximum that could be used and still maintain high precision. A similar strategy could be used with a hand 8-channel pipette but precision would need to be tested. Depending on the manufacturer of the arrays and their reagent requirements, the protocol may differ from what is shown in Table 5 but the basic order of events contained therein should be maintained. As plates are finished being assembled and ready for qPCR analysis, they are sealed and briefly spun down and stored at 4˚C until run on the qPCR instrument. Plates are run

back-to-back to minimise the total run time per day. After each plate is run, it is good practice to make a quick check of the amplification curves for the plate to make sure the overall shape of the curves looks similar from plate-to-plate. If there is a plate where the curves are not similar to the others, that plate, (sample) should be dropped from further analysis on technical grounds. Since the cause may be due to a problem with the reagent setup, it is important to ensure that every sample is being treated uniformly.

miRNA Array Plate Setup

Prethaw Exiqon 2X SYBR PCR master mix on ice, store remaining master mix at 4˚C

1) Add 2,180 µl (2.18 ml) of nuclease-free H_2O to 1-15 ml tube per sample (2)
2) Add 20 µl cDNA sample 1 to the first tube
3) The final volume in this tube is now 2,200 µl (2.2 ml), keep on ice
4) Repeat for 1 other sample (cDNA reactions) on day 1, (1-C & 1-T)
5) Repeat for 6 cDNA samples on day 2 (3-C & 3-T)

Exiqon qPCR Protocol

6) Spin down 1 mouse/rat miRNA panel plate/sample, briefly
7) Remove the seal on 1 plate
8) Add 2.2 ml Exiqon 2X miRNA SYBR Green PCR MM to 2.2 ml diluted sample cDNA
9) Mix gently by inverting the tube, spin down
10) Add 540 µl of complete PCR MM to each of 8 wells on a deep 96-well plate (Column 1)
11) Add 10 µl/well to the plate using the Biomek2000 program "miRNA_384_Col 1" program
12) Put an optical seal on the assay plate and spin down
13) Run the plate on the LC480 using the Exiqon miRNA cycling program:

95˚C - 10 min
45 cycles* of:
95˚C - 10 sec
60˚C - 1 min

14) Repeat for the next plate using program "miRNA_384_Col 2"
15) Day 1 run 2 plates (1T & 1C) (and RT rxns)
16) Day 2 run remaining 6 samples and plates (last one runs after hours)
17) Setup using B2000 programs "miRNA_384_Col 3 through miRNA_384_Col 8"

*** - 45 run cycles are required on the LC480 for second derrivative Cq determination through 40 cycles**

Table 11.5 - miRNA Array Plate Setup

Following data collection on the qPCR instrument for all samples, the data need to be exported from the instrument. First, however, consideration needs to be made for how the Cq values are to be determined for the assays on each plate. For some instrument models, there are algorithms in place for determining Cq values without investigator intervention. These algorithms use the shape of the amplification curves to calculate Cq values for each sample on the plate. However, if the threshold-type method is used to determine Cq values, it is critical to have a common assay (interplate calibrator), preferably three technical replicates, on each plate. The mean of the Cq values for this calibrator assay can be used to determine a threshold value so that the mean Cq values for the calibrator assays are the same for each plate. This strategy cannot guarantee uniformity for all plates but it is far superior to choosing a single threshold value for all plates without any experimental basis. Once the Cq values have been determined for each sample plate, data can be exported in the format required by the software being used for data analysis.

Data Analysis

The analysis of qPCR screening data is significantly more complex than the comparatively more simplistic analysis performed for a standard qPCR experiment bearing one or a few transcripts per plate. For this reason, I highly recommend that software that can handle large data sets from qPCR arrays be

used. There are two excellent programs on the market capable of performing data analysis for all aspects of any qPCR experiment, GenEx from Multid and qBase+ from Biogazelle. My example comes from a GenEx analysis workflow but something similar would work using qBase+. Analysis software is available from SA Bio/Qiagen in the form of Excel workbook macros for their data analysis and Life Technologies/Thermo Fisher have software for data analysis as well. However, I prefer a more robust software package that gives the end-user maximal flexibility and that has all the statistical analyses required for any qPCR experiment, include array screens.

The first challenge is to get all the Cq values from each plate/sample into the software with the correct annotation. For a standard qPCR experiment with a few plates, this is not a daunting task and follow-on data analysis can be performed using copy/paste of data to new sheets in an Excel workbook and applying the appropriate formulas. Data from a screen, however, is not so simple. The following description will use the GenEx software as a model but keep in mind that a similar workflow would be possible with qBase+. Recall we have only eight plates of data for four control and four tumour samples with 384 data points on each plate. All successive screens done had two 384-well plates per sample making data organisation that much more complex. GenEx uses a Wizard for importing the plate layout from the array manufacturer, importing data for each sample and adding appropriate labels for each sample. Without the Wizard, just getting the data into the software would be a Herculean task. I will not explain each step in detail as those would be specific to GenEx only but I will give the basics of what is done at each step. Make sure that each sample is appropriately named and that they are in an order than makes sense to you, in this case the control and tumour samples grouped together and labeled appropriately.

There are two main steps in the analysis of qPCR screening data. The first is to process the data so that what remains is ready for statistical analysis. The second step is to determine which miRNAs are changing significantly using the Student's t-Test. Although other statistical tests could be performed, the validity of many of the miRNA assays is questionable and those data will bias the results. Those tests are only meaningful when a finite number of assays with defined parameters are run on the samples in a post-screen validation experiment.

Data processing

Figure 11.7 has a diagram outlining the steps described below.

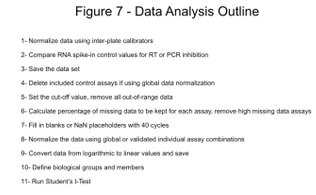


Figure 11.7 - Outline of the data analysis workflow utilising GenEx analysis software.

1- Use the mean values of the inter-plate calibrators to normalise the data among all plates using a test within the software. This will normalise any technical differences among the plates.

2- If a spike-in control was run during cDNA synthesis to test for possible RT/PCR inhibition, there should be a test that will tell you whether these assays on each plate pass analysis. Unless one or more of the plates fails miserably, continue on with the analysis but keep a record of any plates that failed for consideration at the end of analysis.

3- Save a copy of the data set at this point in case you want to come back to this data set as a starting point.

4- Control assays intended for use in data normalisation are provided on each plate. In this case there are non-miRNA, small ncRNA assays on the plate as well as some miRNA assays found in multiple tissue sources. We will be using Global data normalisation so it's best to delete these assays from the data set so they don't bias the normalisation process. You can come back to the saved data set above if you wish to test these assays for their suitability as data normalisers in a follow-up analysis.

5- Next, choose a cut-off cycle value below which the data will be assumed to be invalid for quantification. Since we know nothing about the limit of quantification (LOQ) for any of the assays in the array, this value is somewhat arbitrary. Exiqon recommends a value of 37 cycles, which is high. For an mRNA screening array, 35 cycles would be a more appropriate value. The cut-off value for any assay is determined by the PCR efficiency. By choosing 37 cycles, the assays with low PCR efficiencies are included in the analysis. However, for those assays with a high PCR efficiency, such a high cut-off value will keep data that would normally be excluded in a single assay experiment. For example, at 100% PCR efficiency a single template copy would theoretically be detected at 33 cycles.

By performing the cut-off analysis, the software will eliminate all assays containing either Cq values that are all higher than 37 cycles or where there is no data for any sample. It will also eliminate individual values for assays that have Cq values above 37 cycles or no data and replace it with a NaN. The NaNs are placeholders that will be removed in another step.

6- Next a decision has to be made when to discard assays that have missing data above a certain percentage of the total number of data points where each data point represents one sample. In our case we should have eight data points per miRNA assay. What you want to avoid is eliminating miRNAs where one biological group has a signal and the other has none. That would, of course, be an ideal result. Set the minimum number of acceptable missing data samples to 50%. Any miRNA assay with more than half of the data missing will be eliminated from the data set. To determine this cut-off value for data sets with different sample numbers, divide the number of samples from the smallest biological group (if the number/group is not equal) by 2 and round up if necessary. Divide that value by the total number of samples in all groups, multiply by 100 and use that percentage value as

the cut-off. For this screen we have eight samples in both groups/ 2 = 4, 4/8 = 0.5 x 100 = 50%.

7- The data set still has cells with no value which have to be filled in before we can proceed. There is no way to know why these cells have no value while there are acceptable Cq values (below the cut-off) for other samples for the same miRNA assay. Replace these blanks or NaN placeholders with a value of 40 (cycles) to represent the lowest possible value.

8- Next step is data normalisation. The one real advantage of running so many different miRNA (or mRNA) assays in a qPCR assay array screen is that you can use the mean of all values to normalise the data set (Mestdagh et al., 2009). Run the Global normalisation protocol using only assays where the measured Cq values are ≤30 cycles. You want to normalize the data using cycle values for valid data points only.

9- It's a good idea to save the data file at this stage as well. The data need to be converted into relative quantities. As we want to get true comparative relative values from the t-Test later on, GenEx allows you to set the comparison to nothing. Cycle numbers will be converted to relative values at this step.

10- Finally, you must recall that cycle data are in log format. That is why you subtract instead of dividing when performing a ddCq calculation. The data are converted to a linear value by using a Log2 conversion. Only linear data are normally distributed. Save this final preprocessed data set file and export the data for statistical analysis.

Statistics

1- In GenEx you have to define biological group names and which samples belong in which groups at this stage. There is an opportunity to define members of each group in the import wizard but it can be done here as well. Other programs may handle this issue differently but it has to be done before the final statistics are determined.

2- Run the Student's t-Test on the final exported data set. The miRNAs with the lowest p-Values in a Tumor versus Control analysis (2-tailed, unpaired samples) are shown in Table 6. MicroRNAs with p-Values less than 0.05 are highlighted in yellow.

3- The data for the first sample, miR-207 looks pretty good. That is, the linear fold change is 4.4 and the p-Value is 0.00273. However, GenEx calculates what the p-Value has to be for significance based on the entire data set and in this case, it would have had to be lower than 0.0008. Therefore, although the p-Value is way below 0.05, the usual default value for significance, it is still not low enough to be truly significant in this data set. Just as GAPDH or 18SrRNA are not always appropriate for use as data normalisation assays for all experimental conditions, a p-Value less than 0.05 is not always significant for all data sets, a fact that appears to be unknown by many in the research community. Having said that and taking into account that not all

the data may be valid, this miRNA is a prime candidate for follow-on validation testing.

4- The Normality test column for both biological groups has Not Determined rather than Pass or Not Pass. This is because this statistical test requires a minimum of seven samples per biological group. For Exiqon plates, sold in groups of four, using eight samples minimum per biological group will provide enough data for this test. In this instance, we have no idea how variable the values were for either biological group. Either group failing this test for any miRNA would nullify significant Fold Changes and p-Values. Not having this information means we are not sure how valid the values we do have are, in fact.

5- There are some intriguing results in our candidate list. How to proceed from here? The best approach is to select a subset of miRNAs from this list that have the greatest probability of having an influence on the biological phenomenon being investigated, in this case osteosarcoma verses the control samples. By testing these candidates with larger biological groups using individual miRNA assays in a validation experiment, we can find out if they are in fact true regulatory elements within our biological system as described below. An alternative validation plan would involve using a different technique such as microarrays or NGS which would have been an equally valid approach.

Table 11.6 GenEx Student's t-Test Report (part 1)

(Tumor) vs (Control)	Normality test (Tumor)	Normality test (Control)	Fold change	Difference (log scale)	P-Value
rno-miR-207	Not Determined	Not Determined	4,4	2,13	0,00273
mmu-miR-675-5p	Not Determined	Not Determined	7,1	2,82	0,00462
mmu-miR-297c	Not Determined	Not Determined	5,1	2,35	0,00891
mmu-miR-743b-5p	Not Determined	Not Determined	4,9	2,29	0,01139
mmu-miR-130b	Not Determined	Not Determined	3,2	1,67	0,01207
mmu-miR-197	Not Determined	Not Determined	9,2	3,20	0,01262
rno-miR-409-5p	Not Determined	Not Determined	-4,5	-2,16	0,01366
mmu-miR-687	Not Determined	Not Determined	6,5	2,71	0,01372
mmu-miR-742	Not Determined	Not Determined	3,5	1,80	0,01653
mmu-miR-381	Not Determined	Not Determined	2,2	1,15	0,01742
mmu-miR-361	Not Determined	Not Determined	-1,4	-0,52	0,01791
mmu-miR-871	Not Determined	Not Determined	25,6	4,68	0,01979
mmu-miR-9	Not Determined	Not Determined	17,7	4,15	0,02258
mmu-miR-685	Not Determined	Not Determined	1,8	0,82	0,02372
mmu-miR-146b	Not Determined	Not Determined	-4,2	-2,09	0,02799

Table 11.6 GenEx Student's t-Test Report (part 2)					
mmu-miR-345-3p	Not Determined	Not Determined	-1,9	-0,92	0,02805
mmu-miR-181d	Not Determined	Not Determined	3,3	1,70	0,03165
mmu-miR-874	Not Determined	Not Determined	3,1	1,63	0,04057
mmu-miR-20a	Not Determined	Not Determined	1,9	0,90	0,04199
mmu-miR-181c	Not Determined	Not Determined	2,3	1,23	0,04516
mmu-miR-139-3p	Not Determined	Not Determined	2,3	1,23	0,04665
rno-miR-346	Not Determined	Not Determined	-2,5	-1,30	0,04684
mmu-miR-217	Not Determined	Not Determined	2,4	1,24	0,04709
mmu-miR-380-3p	Not Determined	Not Determined	-6,2	-2,64	0,04721
mmu-miR-34a	Not Determined	Not Determined	-1,9	-0,95	0,04759
mmu-miR-449a	Not Determined	Not Determined	-10,9	-3,44	0,04760
mmu-miR-362-5p	Not Determined	Not Determined	-1,6	-0,65	0,04973
mmu-miR-682	Not Determined	Not Determined	2,3	1,20	0,05085
mmu-miR-200a	Not Determined	Not Determined	-3,1	-1,65	0,05094
mmu-miR-297b-5p	Not Determined	Not Determined	5,0	2,31	0,05285
rno-miR-146b	Not Determined	Not Determined	-3,6	-1,85	0,05375

Table 11.6 GenEx Student's t-Test Report (part 3)					
mmu-miR-670	Not Determined	Not Determined	4,2	2,08	0,05378
mmu-miR-452	Not Determined	Not Determined	2,1	1,05	0,05442
mmu-miR-19b	Not Determined	Not Determined	1,5	0,57	0,05465
mmu-miR-680	Not Determined	Not Determined	2,9	1,52	0,05794
Highlighted samples included in post-screen validation experiment					

Section 6

Performing a qPCR Validation Experiment

1. Assay Rationale and Setup
2. Analysis

Assay Rationale and Setup

The purpose of the screen is to find candidates for more thorough testing. This is true of any screen regardless of the technique employed. Unfortunately, many investigators spend their time and resources on proving the validity of the screening results rather than testing them in a proper post-screen validation experiment. Validation experiments can use a different technique from the screen, even totally different assay sources. This is certainly true of a qPCR screen as one could use the induction or depression of protein expression following the knockdown of selected miRNAs, for example. In this case we decided to use qPCR again as it was the fastest, least expensive method that the Core lab could perform for the investigator. The next question was should we use Exiqon assays or ones from another vendor? We stayed with Exiqon assays as this assay format has the advantages of using LNA bases in the primers for normalising annealing Tm values and greater template specificity. Locked nucleic acid bases or LNAs (Exiqon holds this patent) have the advantage of imparting a higher Tm per base than standard bases. Thus, the primers can be much shorter and have the same Tm value compared to primers with standard bases. Further, since fewer bases make up the primer, they have higher template specificity as a single mismatch can be much more disruptive to primer annealing to the target sequence compared to a standard nucleotide base. The method used for synthesising cDNA

is universal and avoids any issues with 3'-base extensions added to processed microRNAs as found by sequencing experiments (Wyman et al., 2011). This decision was also based on limited in-house comparison studies using assays from multiple vendors of miRNA assays with Let family members as assay targets. This is not to say that miRNA assays from other vendors do not work, they certainly do, but rather why we continued to use the Exiqon assay system.

Rather than focusing on just the miRNA candidates from the screen, the investigator decided to test miRNAs that had been implicated by other studies in osteosarcoma using individual miRNA assays. While all the miRNAs in this subset were in the screen, only two of them were in the 'hit' list. Although this isn't a perfect example, it does present an opportunity to illustrate how a validation experiment is setup and performed while covering the most important aspects of the MIQE Guidelines.

Table 11.7 shows a comparison of the details from this validation experiment with those of the screen described above. LOQ and PCR efficiencies for each miRNA assay were obtained by using the PCR products from the screen for each target assay. Using those as template, 10-fold dilutions over 7-logs in molecular-grade, 100 ng/µl RNase-DNase-free E. coli tRNA (as carrier) were made starting with a 1/10,000 dilution of PCR product in E. coli tRNA. Note: amplified PCR product will contaminate your work area if not handled carefully. It is best to open the plate cover and make the 1/10,000-fold dilution of PCR product far, far away from your workbench using pipettes, tips and dedicated carrier solution that is not used to set up validation qPCR experiments. Due to the ligation step prior to RT within the Exiqon RT reaction, it is not surprising that there were no signals found in a test no-reverse transcriptase control experiment. There were also no signals found for any sample in a no template control test. Both of these were conducted separately from the validation experiment described below. The RNU1A1 assay used for data normalizationisation was determined from the screening plate data using geNorm analysis within the GenEx software. Compared to all the other potential ncRNAs on the plate array, this was the best choice. geNorm analysis also paired RNU5A with RNU1A1 for optimal data normalizationisation but the investigator was only willing to have a single assay for data normalizationisation. Such is the frustration of being a core lab director. I should also note here that non-coding RNAs are not preferred for qPCR data normalisation for either mRNA nor miRNAs but the latter had not been established at the time this study was performed.

Setting up a validation plate bearing 12 ncRNA qPCR assays for 16 samples (eight per biological group), using the least amount of sample, reagents and time requires some planning. The best setup is having all samples and assays on a single plate that is

assembled and run at the same time. If the 16 samples are run in duplicate for each of the 12 assays, you can see that this will just fill a 384-well plate (16 rows x 24 columns). Other sample/assay combination robot programs were setup at the same time for future validation experiments. For experiments with larger sample sizes, multiple plates would be required. In this instance, all plates have to be setup and run on the qPCR instrument within the same day.

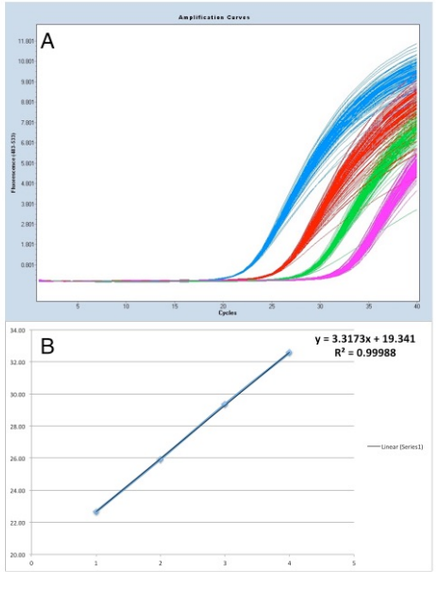
Next, how to assemble the components? A standard qPCR experiment has the assay components included in the PCR master mix and the sample cDNA is then added. That is the only practical way to assemble a PCR plate when there are a large number of samples to be run for each target assay. However, in this case an alternative strategy presented itself, adding the template cDNA to the PCR master mix and then adding the assay components to each cDNA plus sample master mix. In this way, the template would not have the technical variability found in the standard assay setup described above. Slight changes in the amount of assay components added per reaction will have less effect on the final Cq values than variation in the amount of cDNA added per reaction. A Biomek 3000 liquid handling robot with an 8-channel tool was available so a program to combine all the components was planned for 16-samples in duplicate and 12-ncRNA assays. The same plate setup strategy could be made using an 8-channel pipette set up by hand. Before using any robot program, it has to be tested for accuracy and precision. qPCR data from the Biomek 3000 for a validation program designed to test four different qPCR assays against 48-samples, in duplicate, are shown in Figure 8. In this experiment, each sample block of 96-wells contained one dilution of a four-log template dilution series with master mix containing purified synthetic oligonucleotide bearing the assay amplicon sequence as template in place of cDNA for the human Versican qPCR assay. Primer and probe were placed in each of the four different assay positions. As you can see, the data are very tight for all 96-samples per template dilution when run on the LC480. The linear regression is also shown in Figure 11.8 and the mean data for all 4-dilutions are shown in Table 11.8. A similar quality control (QC) experiment was performed for layouts for 16 samples x 12 assays and 32 samples x 6 assay programs. The number of assays that can be run with 32 and 48 samples is proportionally reduced to fit a 384-well plate but the setup for those experiments is essentially the same as shown for 16 samples.

4-Log Dilution Series		
48 Samples/Dilution		
Dilution	Mean Cq	StDev Cq
1	22,66	0,09
2	25,94	0,16
3	29,36	0,14
4	32,58	0,25

Table 11.8 - 4 Log Dilution series

Figure 11.8 - Biomek 3000 quality control testing of a validation program designed to test 48 samples in duplicate against four different qPCR assays. Purified oligo template for the human Versican transcript assay was diluted in 10-fold decrements over a four log range. Each template dilution was added to sufficient qPCR master mix, sans primer/probe, to put 6 µl/well in 96-wells for each dilution. A primer/probe mix (400/100 nM, resp.) was added to the source plate in each of the four assay positions. Four microliters of primer/probe mix were added per well. The assembled plate was covered by optical film, briefly spun and run on an LC480 instrument. A- amplification curves for 96 wells for each of the 4 template dilutions; B- regression analysis of the mean Cq values for each block of 96-well values.

Figure 8- 4-log Template Dilution Series- 48 Samples in Duplicate



The first step in the validation process was to make cDNA from all 16 samples using duplicate RT reactions for each sample so that any variability at the RT step would be included in the analysis. Figure 11.9 shows how each sample was laid out in a 96-well full skirted PCR plate and Table 11.9 shows how each sample was diluted prior to cDNA synthesis. Due to the tapered geometry of full-skirted PCR plates, we found that they make the best source plates for robotics. The 16 samples were set up by hand and processed for cDNA synthesis using a thermocycler (MJR Dyad, 96-well block) using Exiqon reagents and protocol for individual assays. The reagent setup for cDNA synthesis is shown in Table 11.10. The cDNA template for each sample for all 12 ncRNA assays was mixed with an equal volume of PCR master mix large enough for addition to the 24 wells for each sample. Table 11.11 shows the dilution setup for each primer assay and Figure 11.10 shows how the diluted assay components were arrayed in a 96-well PCR source plate. Table 11.12 shows the protocol for dilution of the cDNAs and how they were added to the Exiqon PCR master mix. The robot added 6 µl of cDNA/PCR master mix per well for each sample on the 384-well PCR assay plate followed by 4 µl of diluted primer mix for each assay. The final layout on the 384-well plate can be seen in Figure 11.11. The plate was sealed, spun down and run using the cycling conditions recommended by Exiqon for individual assays for 45 cycles on a Roche LC480 real-time qPCR instrument. Final Cq values were determined using the second derivative algorithm. The data were exported as a text file. Representative Cq values for 10 of the 12 assays are

represented by mmu-miR130b and can be seen in Table 11.13. The corresponding amplification curves are in Figure 11.12. The data for assays mmu-miR129-5p and mmu-miR670 were below their respective LOQ values and were not processed further.

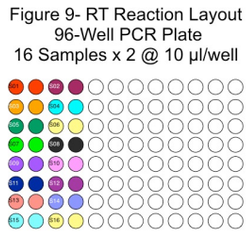


Figure 11.9 - RT reaction plate layout. Order and placement of each of 16 samples, in duplicate, for the validation experiment on a 96-well PCR plate. cDNA was synthesised following the Exiqon protocol for individual assays. Note that odd numbered samples are in the first two columns, even numbered samples in the next 2 columns. This is because an 8-channel pipette dispenses every other well in a 384-well plate thus ensuring the cDNA samples are in linear order in the 16-row, 384-well qPCR plate.

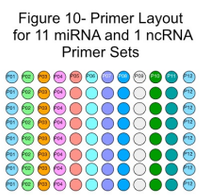


Figure 11.10 - Exiqon primer layout for 11 miRNA and one ncRNA assays. Sufficient primer volumes for 32 wells of each assay were added to eight wells of the source plate per assay in one column of the plate. The well labels are generic.

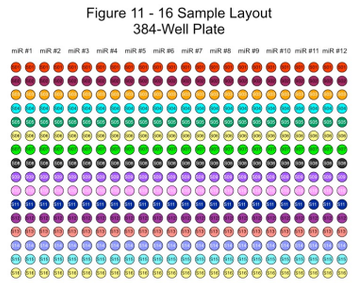


Figure 11.11 - The final qPCR plate layout for 16 cDNA samples run against 12 different miRNA or ncRNA assays. Each sample runs in duplicate across the 24 rows of the plate. Each assay primer pair was added to two columns of 16 wells each to complete the matrix.

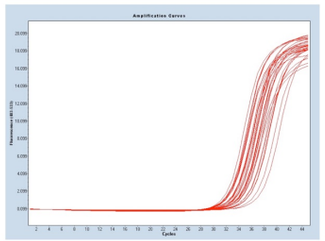


Figure 11.12 - Amplification curves for the mmu-miR130b assay for the 16 samples, 32 reactions from the post-screen validation experiment. These curves are representative of the data obtained for the other nine assays that had sufficient data for further analysis.

Table 11.9 - Dilution of RNA samples for RT Reactions						
Validation Experiment						
1- Make RNA dilutions into Eppendorf Lo-Bind tubes						
		RNA Sample Dilutions for miRNA Screening				
		(ng/µl)	(µl)	(µl)	(µl)	(ng/µl)
Sample Name	Sample ID	RNA Conc	Nuc-free H2O	RNA Vol	Final Vol	Final RNA Conc
C-1	#1	120	48	2,3	50	5,5
C-2	#2	120	48	2,3	50	5,5
C-3	#3	130	48	2,1	50	5,5
C-4	#4	240	98	2,3	100	5,5
C-5	#5	200	97	2,8	100	5,5
C-6	#6	200	97	2,8	100	5,5
C-7	#7	200	97	2,8	100	5,5
C-8	#8	200	97	2,8	100	5,5
T-1	#9	200	97	2,8	100	5,5
T-2	#10	200	97	2,8	100	5,5
T-3	#11	200	97	2,8	100	5,5
T-4	#12	200	97	2,8	100	5,5
T-5	#13	112	48	2,5	50	5,5
T-6	#14	263	98	2,1	100	5,5
T-7	#15	120	48	2,3	50	5,5
T-8	#16	220	98	2,5	100	5,5
2 - Store samples at 4° C						

Table 11.10 - cDNA Synthesis for Validation Samples			
Exiqon RT Reagents			
RT Master Mix		1 Vol	38 vol
Reagent	Final Amount	Volume	Volume
Nuclease-free H2O	*****	4,5	171
5X Reaction Bfr	1X	2	76
RNA spike-in	1X	0,5	19
Enzyme Mix	1X	1	38
	Total (µl)	8	304

Table 11.10 - Exiqon RT Protocol	
1) For each RNA sample (16) need to make 2-10 µl cDNA reactions = 32 total	
2) Label 1 96-well PCR plate for samples #1 - #16, 2 wells/sample (see layout)	
3) Carefully add 8 µl RT Master Mix to each well = 32 total	
4) Very carefully add 2 µl diluted sample RNA 5.5 ng/µl) to each of 2 wells/sample	
5) Run the plate in the MJR Dyad thermocycler as follows:	
	42˚C - 60 min
	95˚C - 5 min
	20˚C - soak
6) Put a heat seal on the RT plate and store at 4˚C until used	

Table 11.11 - Exiqon miRNA Primer Diutions for 16 Sample Validation

Number	miRNA Primers	Number	miRNA Primers
#1	mmu-mir-29a	#7	mmu-mir-125b-5p
#2	mmu-mir-670	#8	mmu-mir-129-5p
#3	mmu-mir-221	#9	mmu-mir-9
#4	mmu-mir-130b	#10	mmu-mir-187
#5	mmu-mir-222	#11	mmu-mir-130a
#6	mmu-mir-100	#12	RNU1A1

Exiqon Primer Mix	xxx-xxx-xxx		55 vol
Nuclease-free H2O	*****	3	165
Exiqon Assay Primer Mix	1X	1	55
	Total (*µ*l)	4	220

1) Add 27 *µ*l diluted miRNA primer/well in 1 column each (see layout) of a 96-well PCR plate

Table 11.12 (part 1) - 10-Fold Dilution of Validaton cDNAs

1) Add 90 *µ*l nuclease-free H2O to each cDNA reaction (= 32 total)

2) Mix well after each addition

3) Tap spin plate

4) store on ice until used

5) Store at -20˚C for long term

B3K Setup for qPCR miRNA Assay Validation

Thaw Exiqon SYBR PCR MM on ice

Thaw miRNA primer mixes on ice

			1 Vol	18 Vol
Initial Amount	Reagent	Final Amount	Volume	Volume
1:10 Dilution	cDNA	1:100	1	18
2X	SYBR PCR MM	1X	5	90
			6	108

Table 11.12 (part 2) - B3K Setup for qPCR miRNA Assay Validation
1) Add 90 µl Exiqon 2X SYBR PCR MM/well to columns 1 & 2 on a 96-well deep plate
2) Add 18 µl diluted cDNA to each well, 1 well/sample as per the layout
3) Mix well with pipetter after adding cDNA & on plate mixer 1 min @600 RPM
4) Use this plate as cDNA source for all 12 primers (miRNA assays)
5) Put the cDNA/SYBR MM, Primer & Roche LC480 plates plus 2-50 µl tip boxes on the B3K deck
6) Run on the B3K using the miRNA Validation program for 16 samples

Table 11.13 (part 1) - LC480 Data for mmu-miR130b

Include	Color	Pos	Name	Cp	Mean Cp	StDev Cp
VRAI	255	A7	C-1	35,76	36,46	0,98
VRAI	255	A8	C-1	37,15		
VRAI	255	B7	C-2	33,15	33,05	0,15
VRAI	255	B8	C-2	32,94		
VRAI	255	C7	C-3	35,75	35,51	0,35
VRAI	255	C8	C-3	35,26		
VRAI	255	D7	C-4	35,26	35,90	0,90
VRAI	255	D8	C-4	36,53		

Table 11.13 (part 2) - LC480 Data for mmu-miR130b

Include	Color	Pos	Name	Cp	Mean Cp	StDev Cp
VRAI	255	E7	C-5	34,81	34,45	0,51
VRAI	255	E8	C-5	34,09		
VRAI	255	F7	C-6	34,16	34,93	1,09
VRAI	255	F8	C-6	35,70		
VRAI	255	G7	C-7	33,26	33,50	0,33
VRAI	255	G8	C-7	33,73		
VRAI	255	H7	C-8	34,31	34,38	0,09
VRAI	255	H8	C-8	34,44		
VRAI	255	I7	T-1	34,21	34,03	0,25
VRAI	255	I8	T-1	33,85		
VRAI	255	J7	T-2	32,16	32,25	0,12
VRAI	255	J8	T-2	32,33		
VRAI	255	K7	T-3	33,76	33,70	0,09
VRAI	255	K8	T-3	33,63		
VRAI	255	L7	T-4	31,75	31,98	0,33
VRAI	255	L8	T-4	32,21		
VRAI	255	M7	T-5	33,78	33,37	0,58
VRAI	255	M8	T-5	32,96		
VRAI	255	N7	T-6	32,44	32,35	0,13
VRAI	255	N8	T-6	32,25		
VRAI	255	O7	T-7	33,02	32,74	0,40
VRAI	255	O8	T-7	32,45		
VRAI	255	P7	T-8	34,35	34,43	0,11
VRAI	255	P8	T-8	34,50		

Analysis

The final data set from a t-Test analysis plus fold differences for the remaining nine candidate miRNA assays can be seen in Table 11.14. There are five miRNA assays (in blue) that have greater than a two-fold difference between the eight control and eight tumour sample RNAs tested but only two, miR-125a-5p and miR-187, have significant fold-differences with correspondingly low p-values. These two are examples of final candidates worth following up with larger sample sizes and with other assay techniques. If you look at the initial screening data using only 4-samples in each biological group, you will be reminded that miR-9 and miR130b were very promising candidates (Table 11.6). However, in looking at the validation results, neither held up for further study. From the data, a dendrogram and a principal component analysis graph were generated in GenEx but in neither case did the control and tumour samples cluster in 2 separate groups. Group-specific clustering and linkage is the ideal result from these 2 analyses. In the dendrogram, two of the tumor samples clustered with two of the tumor samples and three of the controls clustered with the remaining tumour samples (data not shown). In the PCA, there was no group-specific clustering. This is most likely due to the fact that not all of the miRNA assays differentiated the two biological groups.

Table 11.14 - Student's t-Test - Fold Differences

Validation	8 x 8 - Samples	
miRNA	Fold Difference	p-Value
miR-9	1,18	0,8708
miR-29a	1,95	0,0375
miR-100	2,87	0,0217
miR-125a-5p	4,06	0,0012
miR-130a	2,34	0,0546
miR-130b	1,92	0,0550
miR-187	4,96	0,0001
miR-221	1,84	0,1413
miR-222	2,33	0,0497
RNU1A1 used for data normalization		

Section 7

Conclusions

Once the decision to perform a RT-qPCR screen has been made, the information above should provide a useful outline of how to proceed. The strategy employed can be applied to mRNA screens from any vendor. There will be some vendor-specific step changes for sure, but the basic strategy will still apply. Any screen is a search for candidates that are changing within the biological context being studied. Follow-on validation is critical for arriving at a final list of assays worthy of further study.

The first basic tenet is to keep the experimental workflow as consistent as possible for all samples involved. Each major step in the process must be tested to make sure that everything is working as expected for the qPCR instrument and the remaining equipment being used. The largest variable in any qPCR experiment is the person doing the work. Therefore, only one person should perform each step so that any variability is keep consistent at each step of the process. Not only is it important that each sample be handled uniformly throughout the screening and validation process, but the order in which samples are run must also be taken into account. For any experiment, there is always technical error. Keeping that error to a minimum so that more than the largest biological differences can be measured is the goal.

Secondly, it is critical that enough samples be used for the screen to ensure the best results. Exiqon miRNA screening plates come in packs of four so it makes sense to use sample sizes in multiples of four. For laboratory mice, even though inbred, four samples per biological group were not sufficient. A minimum of seven samples per biological group is required in order to test for normality in the data amongst the group members. If the data are greatly scattered within the members of one or both biological groups, the fold difference between the groups will be meaningless. Without enough members to perform this test, the screen was at a disadvantage statistically. That begs the question how many samples are needed in each group? Some time ago in a clinical trial when RNA samples were low in abundance, the question was asked “how many human samples does it take in a pool to equal the values obtained from running each sample individually”. This was for total RNA samples from the endometrium looking at six different mRNA targets using RT-qPCR. The answer was 20 samples per biological group. In the example shown above, more than four samples per biological group for the screen would have been desired, at least eight. However, only four samples per group passed our in-house RNA quantity and quality measures and the investigator didn’t want to wait months to generate new samples. The ideal number for the screen would have been 8-12 per group and for the validation, over 20 per group to get solid statistical data. As seen in the above examples, there were relatively few candidates found or potentially validated when fewer samples per group were used. As described above, those sample numbers are not always practical so a reasonable middle ground must be found.

Finally, make sure you have a solid plan for data analysis. The example above should point out that a screening experiment is not the same as a standard qPCR experiment. The use of commercially available software is highly encouraged. Every possible step in the process can be easily performed and any qPCR experimental data set can be analyzed.

Acknowledgement

I would like to thank Dr. Stephen Bustin for his assistance in reviewing the manuscript prior to submission.

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Chapter 20

RT and qPCR Troubleshooting - How to Improve Your qPCR Data

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Introduction

The advancement to quantitative real-time PCR (qPCR), which allows for the determination of fluorescence signals already in the amplification of the starting sequence, together with the advanced knowledge of the genome base sequence of organisms, was the starting point for establishing this method as an extremely important detection system.

The method of qPCR often combined with reverse transcription (RT) for quantitative RT-PCR is now a standard method in modern analysis and research laboratories of various industries. The most common applications are gene expression analysis by RT-qPCR, disease diagnostic and management (e.g., viral quantification), food testing (e.g., GMO or allergens analysis) and animal or plant breeding.

This method is very sensitive, so error prevention is very important in order to generate reliable data. The whole workflow of RT and qPCR data evaluation consists of several starting points.

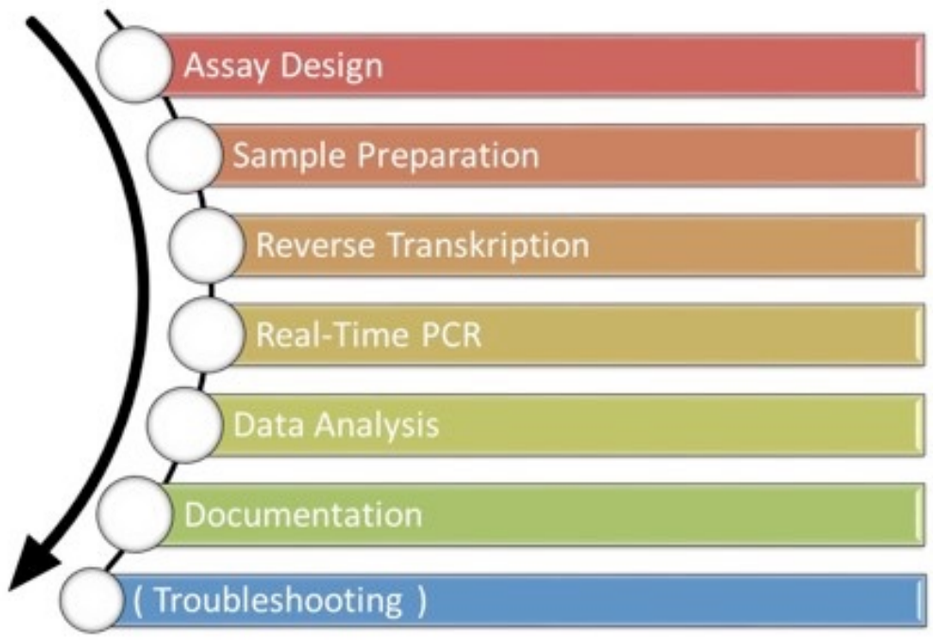
1. Assay design
2. Sample treatment and purification/extraction
3. RT and qPCR
4. Data evaluation

Unfortunately, there are endless possibilities for operator errors and mistakes.

Very often, the sources of these errors remain unidentified.

The first step in any troubleshooting procedure is to check the protocol again and repeat the experiment.

Real-Time-PCR workflow



Experimental Design

The first essential step of an RT or qPCR reaction is the determination of the PCR product and the corresponding primers and probes. The most efficient design for real-time RT-qPCR primers and probes is using software. Most programs include adjustable parameters for optimal properties. These parameters already consider the melting temperature, complementarity, and secondary structures as well as amplicon size and other important factors. Restricting the number of identical nucleotide runs is also recommended. When designing amplicons in eukaryotic targets, choose PCR primers that span at least one exon-exon junction in the target mRNA in order to prevent amplification of the target from contaminating genomic DNA. For the design of gene-specific primers, the following criteria should be fulfilled:

Primer design

- PCR product lenghth qPCR: 75 - 200 bp
- Primer lenght: 18-30 bases
- GC content: ideal 40-60%
- Melting temperature: 55 - 60 °C
- 3'-end: 1-2 G or C for good stability
- Not more that 3 G or C (Mismatching possible)
- Free of secondary structures, repetitive sequences or palindroms
- Concentration: 150 – 200 nM

Sample Preparation and Extraction/Purification

Extraction/Purification

The quality of the template has a direct influence on the assay performance and needs to be considered in the troubleshooting process of qPCR results as well. First of all, tissue sampling and storage is the first potential source of experimental variability. For gene quantification, high quality RNA has to be used, which means that RNA quantity and quality need to be checked very carefully. Degraded or impure RNA can limit the efficiency of the RT reaction and reduce yield; partially degraded RNA may not give an accurate representation of gene expression. Nucleic acid should be extracted and purified from fresh or immediately frozen tissue; the usage of biological replicates (at least 3) is recommended as well. After the sampling process, the isolation of RNA or DNA is the key step in the pre-PCR process. This is necessary so that the extraction is efficient for all samples and the result in a purified nucleic acid (DNA, RNA). The quality needs to be checked on a high-resolution agarose gel (sharp ethidium bromide–stained rRNA bands) and spectrophotometry (A260/A280 = 1.8 and A260/A230 = 2.0). All surfaces of the work space used for PCR preparation should be routinely decontaminated to prevent cross-contamination using a DNA decontamination solution that destroys DNA.

(Reverse Transcription and) Real-time PCR:

1 No amplification

2 Unexpected Efficiency

3 Shifted Cq values

4 Inhomogeneous Replicates

5 Height and Shape of Amplification Curve

6 Unexpected Signals

RT-qPCR is a highly sensitive tool for analyzing DNA or RNA. As the PCR amplifies the target, errors are simultaneously amplified. You can reduce technical errors in PCR reaction setup by standardizing (or robotizing, if possible) and minimizing the number of pipetting steps. Mix cDNA with qPCR reagents, then aliquot a standard volume of this “master mix” into each reaction well containing a standard volume of specific primers. Set up reactions in a clean environment free of dust, preferably under a positive airflow hood. Routinely check for DNA contamination of primer and reagent stocks by performing PCR reactions on no template (water) controls.

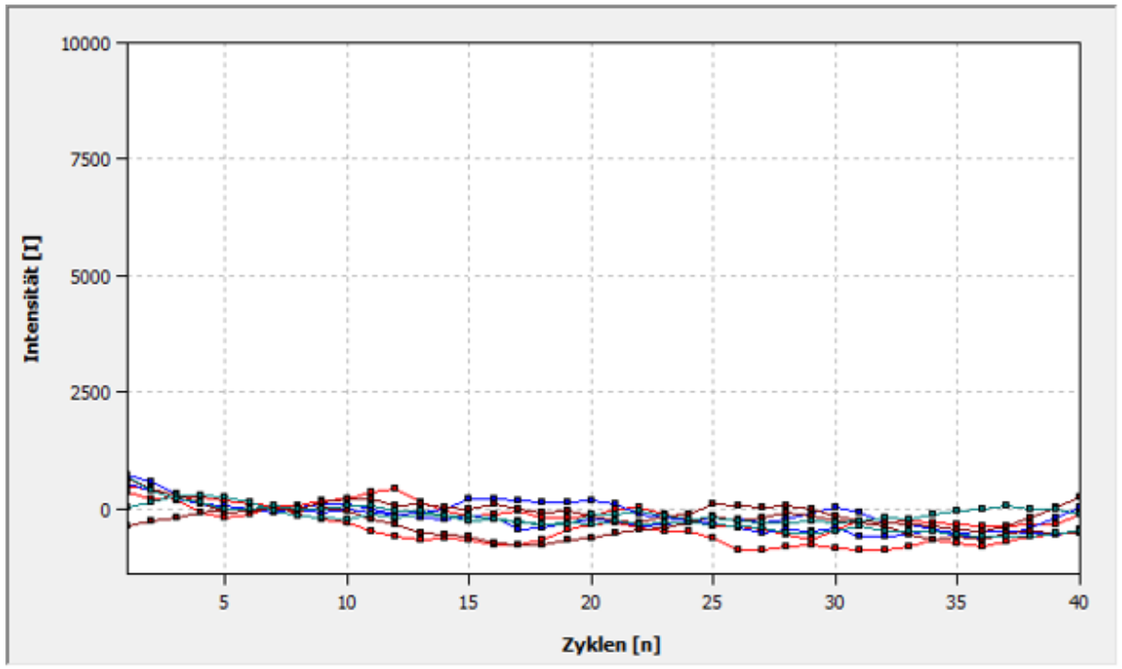
Fachbeitrag Real-time PCR Reproduzierbarkeit

The following symptoms can occur when performing a qPCR:

- no amplification
- unexpected PCR efficiencies
- delayed Cq values
- early Cq values
- inhomogeneous replicates
- height of amplification curve
- unusual curves
- unexpected signals
- primer dimers

1. No Amplification

There are a variety of reasons that no amplification or no signals in the reaction wells can occur. First of all, a repetition of the assay is recommendable; if the second (or third) reaction is not working, you have to check the following things:



- Assay Design:

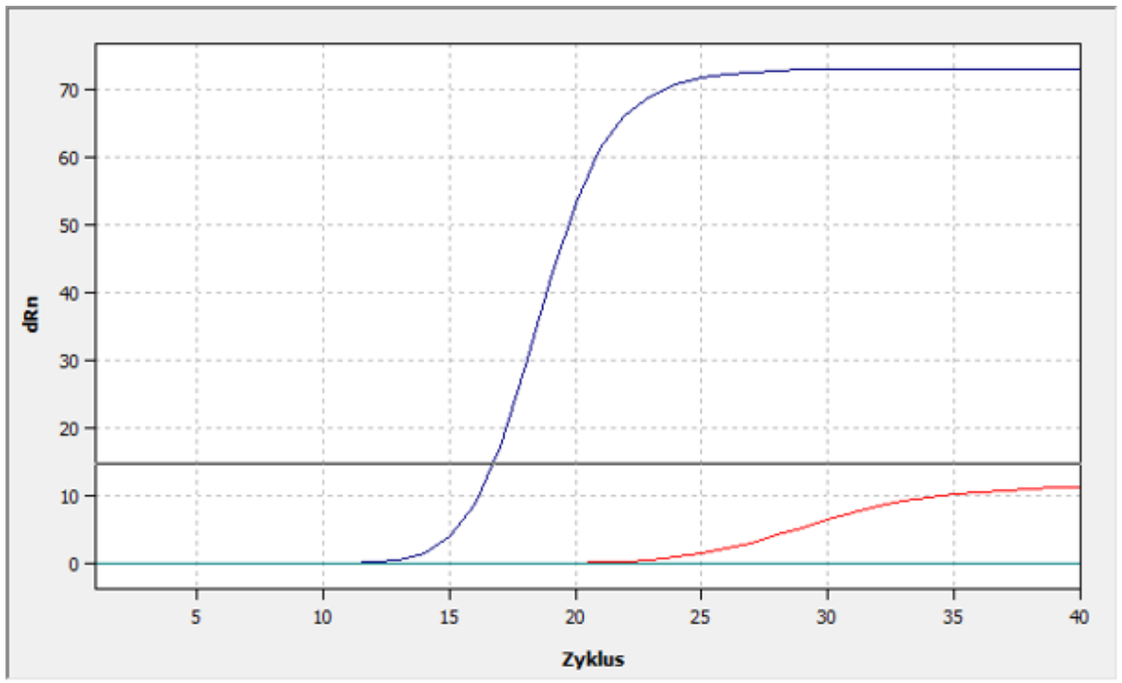
Non-optimized PCR conditions, for instance, the thermal protocol, can lead to the complete failure of the qPCR reaction. To optimize the PCR, it is recommended to perform a gradient reaction with intercalating dye to prove the set up primers. With the gradient function it is possible to determine the optimized annealing temperature. In addition to that, a post-PCR melting step is necessary to distinguish between a specific PCR product or dimer formation. Further troubleshooting can involve optimization of the concentration of PCR components such as magnesium chloride. Using prefabricated master mixes keeps the variability to a minimum wherever possible. A master mix, or mixture of the reaction reagents, should be used when setting up multiple reactions to minimize sample-to-sample and well-to-well variation and improve reproducibility.

- Degraded Template Material:

The extraction of DNA and especially RNA is a very sensitive method and can be very critical. An additional cDNA preparation would be necessary, again starting from RNA isolation. If possible, run a positive control to verify whether the reaction takes place at all or not. When working with RNA, several rules and guidelines need to be followed, such as wearing gloves at all times to avoid contact with RNases, which would lead to degradation. The aliquots must be stored at least at -20°C (or better, at -80°C) because RNA is a more unstable molecule than DNA. It is worth considering DNase treatment, and all adequate controls need to be performed.

- Probe/Dye Quality Issues:

Fluorescent labelled oligonucleotides and intercalating dyes can also be a reason for no amplification in real-time PCR. Fluorophores are light sensitive and can be destroyed/degraded by light exposition. Testing further aliquots of the probe and dye may show a better result.



- Incorrect Dye Detector:

Software and device specifications and settings need to be checked if the dye used can be read out by the filter configuration. Calibration need to be considered as well. Other reasons can be background fluorescence readings on raw data.

Well		Sample name	Sample type	Group	Ct
B4		sample 1	Unknown	Group 1	No Ct
B5		sample 1	Unknown	Group 1	No Ct
B6		sample 1	Unknown	Group 1	No Ct
B7		sample 2	Unknown	Group 1	No Ct
B8		sample 2	Unknown	Group 1	No Ct
B9		sample 2	Unknown	Group 1	No Ct
B10		sample 2	Unknown	Group 1	No Ct

- Curve Does not Cross the Threshold:

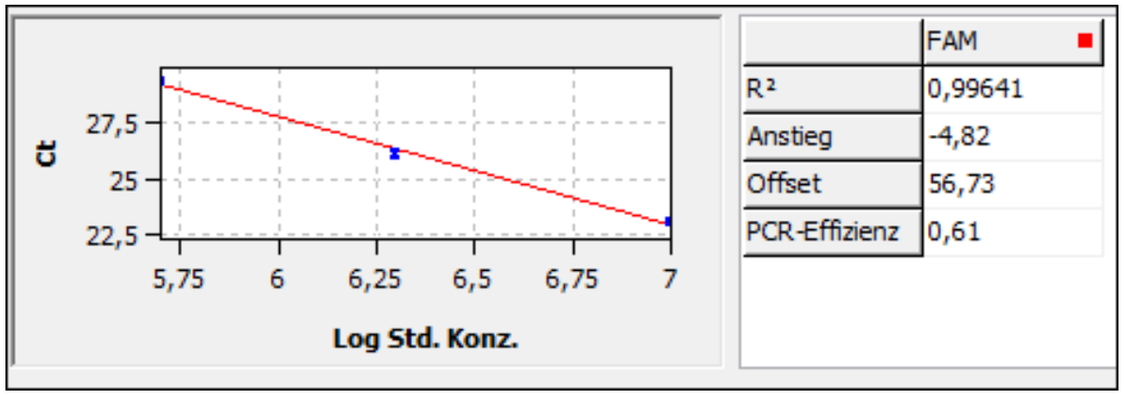
It would be necessary to check the threshold settings; manual setting can help.

2. Unexpected Efficiency

The PCR efficiency indicates whether the DNA is doubled in each cycle, whereas the DNA is amplified 10-fold in 3.32 cycles. To get an appropriate standard curve, at least 3 dilution steps should be used and the use of 3 replicates is recommended. The next important step is to use an appropriate range for standard curves. Standard curves should be prepared for each gene under study for RNA or DNA quantitation (absolute or relative quantitation), or for verification of the efficiencies of the reactions for comparative quantitation (delta-delta-Cq). The standard curve should extend above and below the expected abundance of the examined target. The efficiency (Eff) of the reaction can be calculated using the following equation:

$$\text{Eff} = 10^{(-1/\text{slope})} - 1$$

The efficiency of the PCR should be 90–110% (3.6 > slope > 3.1). A number of variables can affect the efficiency of the PCR. These factors can include length of the amplicon, secondary structure, and primer design, to name a few. Although valid data can be obtained that fall outside of the efficiency range, the RT-qPCR should be further optimized or alternative amplicons designed.



The Efficiency of the Reaction is Poor (<90%)

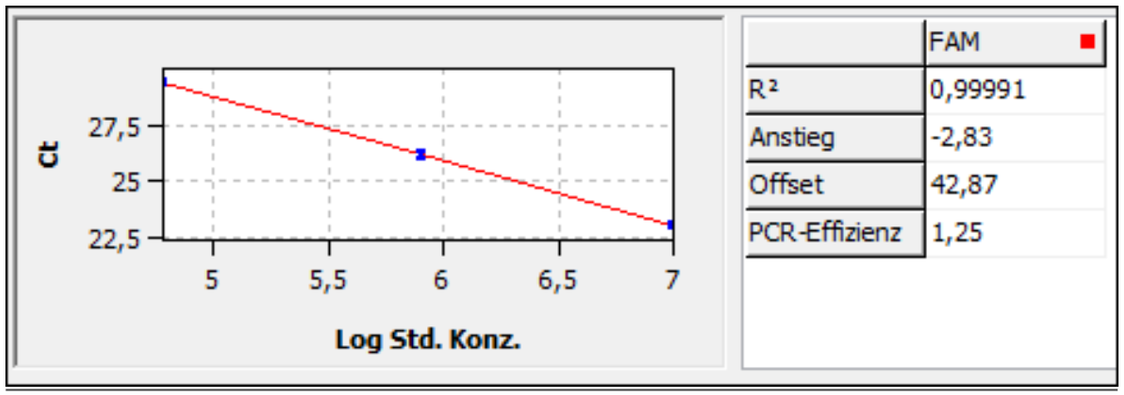
– Incorrect dilutions can cause errors in the standard curve

– Lower fluorescence of dye concentration or the instrument is not calibrated for dye

– Sample inhibition

– Poor primer design

– Dynamic range of the standard curve too small

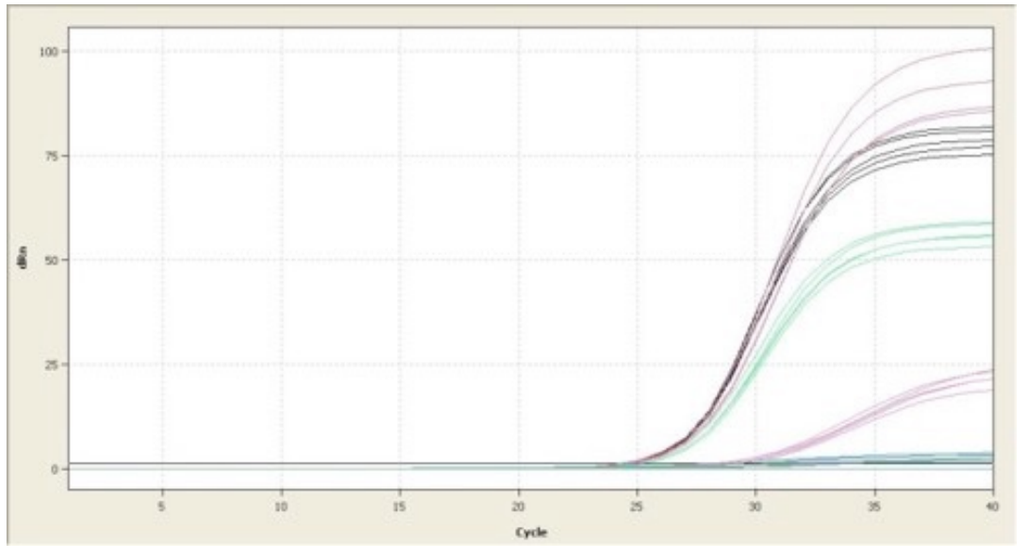


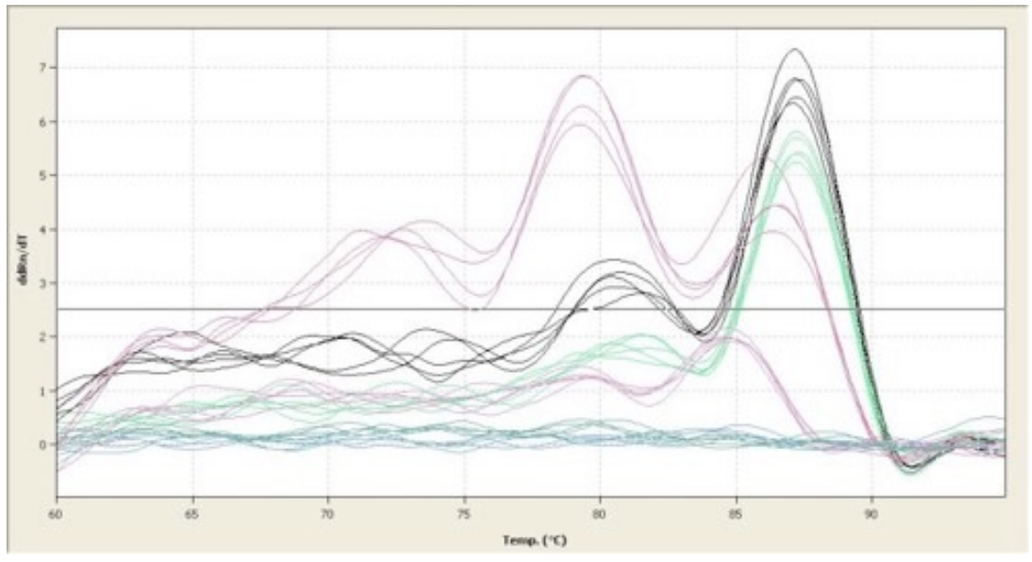
The Efficiency of the Reaction is Higher (>110%)

– Incorrect dilutions causing errors in standard curve

– Genomic DNA contamination (incomplete DNase treatment only)

– Polymerase inhibition (inhibitors affect highly concentrated samples)

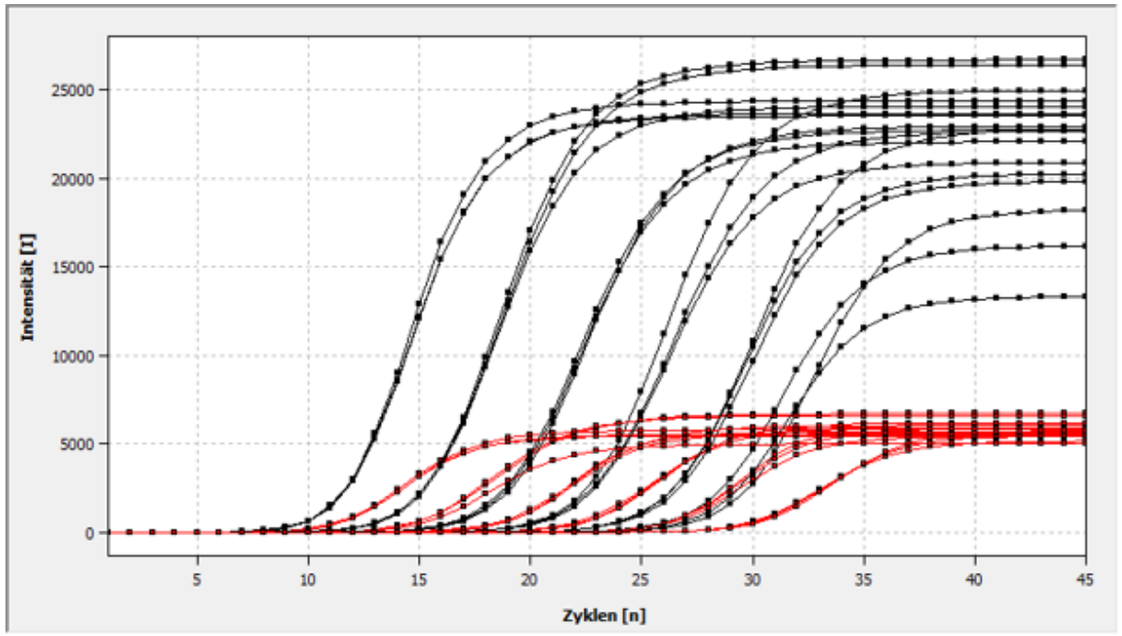




3. Shifted Cq Values

Delayed Cq Values:

Delayed Cq values correspond with a decreased PCR efficiency if all 10-fold dilutions <3.32 cycles produce a lower expression than expected. The reason for that delay could be a sample inhibition. The concentration of inhibitors is highest in the least diluted sample. The higher the dilution factor, the lower the inhibitory effect. To reduce the effect of sample inhibition, it is recommended to perform a 10-fold dilution of the sample and perform the qPCR afterwards (same PCR conditions).



The following criteria can lead to an inhibitory effect:

– chloroform residues from extraction method

– SDS concentrations as low as 0.01%

– phenol

– heparin

– bromophenol blue

– ethanol

Different master mixes from different manufactures can result in a shift in Cq values due to differently performing polymerases in the master mixes.

Early Cq Values:

Ct that is too early can go hand in hand with a template with too high an amount: software cannot distinguish between a background signal and true amplification.

Automated baseline setting is critical when it comes to high template concentration, and manual control is therefore recommended.

Different master mixes can also make a difference.

4. Inhomogeneous Replicates

The most common mistakes are made by pipetting. Precise pipetting is key to receiving accurate and reliable results.

Several other factors can influence the technical replicate performance, (technical) replicates should not be more than 0.5 Cq apart:

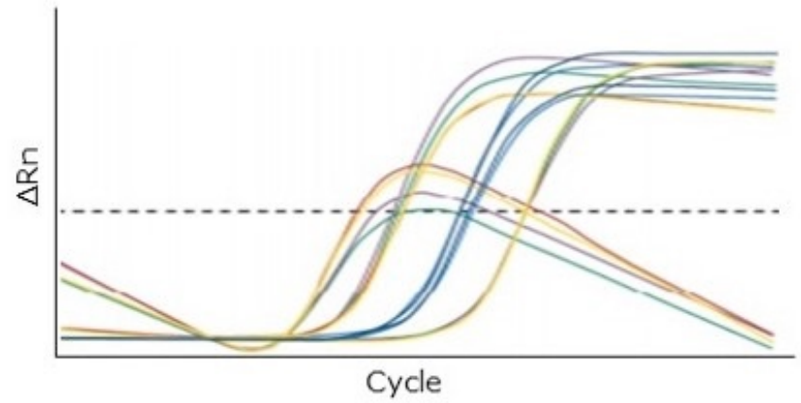
– Bad thermal calibration (heating and cooling of the device is not homogeneous)

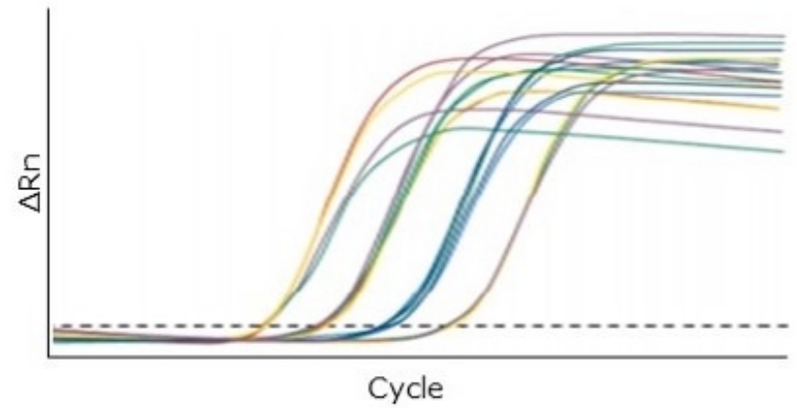
– Denaturation time is too short (fast cycling master mix, consider increasing the denaturation time from 5 to 20 seconds)

– Low copy number

– Wrong baseline settings

– Contaminated NTCs





5. Height and Shape of Amplification Curve

Height:

The height may have an indirect influence on the quantitation because the Ct values are normally used for quantitation and not the endpoint fluorescence height.

A difference in fluorescence heights in the endpoint can result from different intercalating dyes (e.g., the use of EvaGreen® or SybrGreen®). The biggest influence on real-time PCR results from a difference in white and clear plastic ware in several qPCR cyclers.

Appnote Real-time PCR Comparison of Signals

The results of a probe experiment on the variation between singleplex and multiplex is shown here. The height of the

amplification curve is reduced if the target template is amplified in a multiplex reaction (vs. singleplex reaction). It is important to note, however, that the Ct values do not change in the two reactions.

Small and Noisy Curves

A possible reason for small and noisy amplification curves is that the fluorescence signal is too low. New aliquots of probes or dyes can help here, and the concentration needs to be checked. Make sure that high quality HPLC-purified probes (free dyes or unquenched probes will produce high background levels) are used. A check of the instrument is also advisable to ensure that the device is really able to detect the specific dye and that the correct channel is used for detection.

To avoid a problem in the read-out of the fluorescence, a check of the PCR product on an agarose gel is necessary. From that experiment, a clear statement can be made as to whether the whole PCR is not working or only the detection of the fluorescent dye.

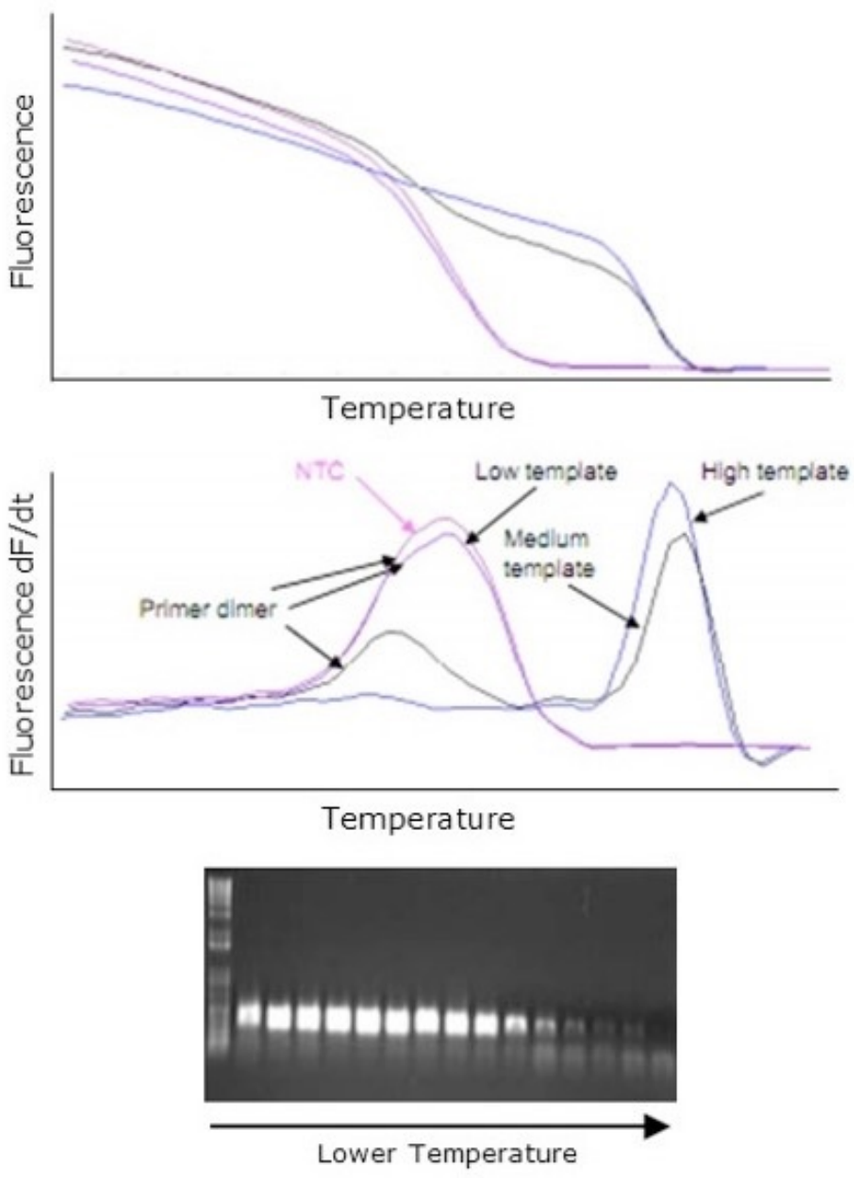
Unusual Curves

The reason for “unusual-looking” curves is mostly sample evaporation. To avoid this sample evaporation, it is recommended to use suitable plate and foil combinations, which fit perfectly into the sample block of the qPCR device. The use of a pre-heated lid with a lid temperature of at least 100°C is necessary to ensure that the contact pressure of the lid works properly. To avoid fluctuation caused by evaporation, ROX can be used as a passive reference to normalize the signal.

A further problem can be that the samples are still not adjusted to the block temperature; using fast protocols can be particularly critical. To achieve better results, set an extended holding time to adjust the temperature, especially when using higher sample volumes. For highly concentrated samples, the baseline setting can also be a reason for the unusual shape of the amplification curve.

6. Unexpected Signals

Positive signals in NTCs can be caused by contamination, primer dimer formation or genomic DNA contamination in RT-qPCR experiments.



Positive NTC (No Template Control)

Positive NTC reactions are often an indication of contamination of reaction components with the target sequence (e.g., water or master mixes). Reducing contamination is mandatory by performing qPCR. To achieve the least reduction possible, some simple rules can be followed: Wearing gloves is essential to protect the samples against RNases from skin and other compounds; using aerosol-resistant filter tips is recommended as well as PCR-quality water, which should only be used for PCR; the experiments can benefit from a PCR sterile workbench with UV decontamination to remove possible template sequences; producing master mixes with sufficient volume for all replicates is recommended; and using non-template control (NTC) to confirm the absence of contaminants is the most important step.

Primer Dimers

Primer dimers can occur in experiments with intercalating dyes, for instance, SybrGreen. The reason is incorrect binding of primers, which leads to unspecific products and these primer dimers interfere with the quantitation of the PCR product. It is necessary to identify unspecific products by performing a melting curve analysis as a post-PCR step. Here, an increase in temperature leads to the release of fluorophore due to the melting of the PCR products. For convenience, we typically view the first derivative (slope) of the actual melting curve data. The pure homogenous PCR products produce a single, sharply defined,

melting curve with a narrow peak. Primer dimers melt at relatively low temperatures and show broader peaks. The resulting graph looks like a chromatogram, with peaks that represent different PCR products.

PCR products that melt at a relatively low temperature are likely primer dimers. PCR products that melt at a higher temperature are likely to be the main PCR product. PCR products that are shorter will melt at lower temperatures.

How to avoid primer dimers:

– Performing a gradient to get to optimized annealing temperature

– Reduce delays in workflow

– Optimize primer concentrations, check for primer complimentary

– Increase the annealing temperature

– Reduce annealing time to 1–5 sec.

– Use hot start

– Reduce number of cycles, e.g., to 40

Positive Reverse Transcription:

RNA that is used for reverse transcription qPCR (RT-qPCR) must be free of genomic DNA. If the primers can bind to the genomic DNA, amplification of the genomic DNA becomes visible, and control without reverse transcriptase is therefore necessary (“No Amplification Control” or NAC) in RT-qPCR experiments. Typically, the NAC is a mock reverse transcription containing all the RT-PCR reagents except the reverse transcriptase. If a product is seen in the NAC, it probably indicates that contaminating DNA is present in the sample and the DNase treatment is incomplete and needs to be repeated.

Summary

To achieve reliable and stable qPCR results, a set of control reactions should be performed with each run:

NC: negative control – checks the specificity of the primers

NTC: control without template – checks for primer dimers and contamination of reagents with template

NAC: control without reverse transcriptase – checks for contamination with genomic DNA when working with RNA

Positive control: checks the functionality of reagents and primers

A successful real-time PCR experiment will have the following characteristics:

Replicates are tightly clustered

Baselines are relatively flat

Dilution series have expected spacing

Plateau height does not matter

Melting curve has one peak per product

Curves are mostly S-shaped

Curves are smooth

Post-run Data Analysis - Settings of Baseline and Threshold

The baseline correction is necessary to remove the background fluorescence.

Possible reasons for that are detector noises of the qPCR devices, different sample amounts or residual fluorescence of the dyes (probes or intercalating dye). All samples are quantified from starting point 0.

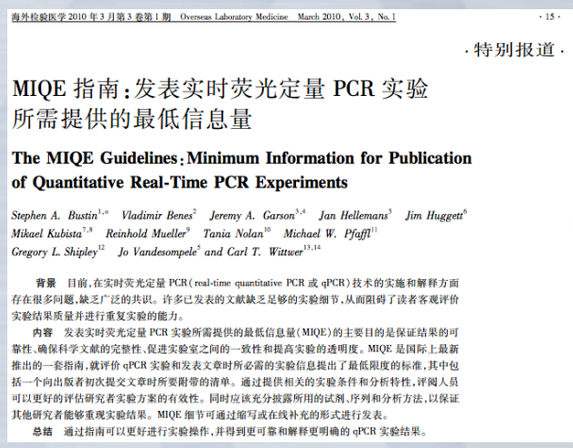
To obtain accurate Ct values, the baseline needs to be set two cycles earlier than the Ct value for the most abundant sample, which means that the baseline stop value should be 1 or 2 cycles before the first amplification occurs. For real-time RT-qPCR data to be meaningful, the threshold should be set when the product is in the exponential phase. Typically, this is set at at least 10 standard deviations from the baseline.

Chapter 21

MIQE Translations

Afif M. Abdel Nour

Chinese



海外检验医学 2010 年 3 月第 3 卷第 1 期 Overseas Laboratory Medicine March 2010, Vol. 3, No. 1 · 15 ·

·特别报道·

MIQE 指南：发表实时荧光定量 PCR 实验所需提供的最低信息量

The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments

Stephen A. Bustin[1,] Vladimir Benes[2] Jeremy A. Garson[3,4] Jan Hellemans[5] Jim Huggett[6] Mikael Kubista[7,8] Reinhold Mueller[9] Tania Nolan[10] Michael W. Pfaffl[11] Gregory L. Shipley[12] Jo Vandesompele[5] and Carl T. Wittwer[13,14]*

背景 目前，在实时荧光定量 PCR（real-time quantitative PCR 或 qPCR）技术的实施和解释方面存在很多问题，缺乏广泛的共识。许多已发表的文献缺乏足够的实验细节，从而阻碍了读者客观评价实验结果质量并进行重复实验的能力。

内容 发表实时荧光定量 PCR 实验所需提供的最低信息量（MIQE）的主要目的是保证结果的可靠性、确保科学文献的完整性、促进实验室之间的一致性和提高实验的透明度。MIQE 是国际上最新推出的一套指南，就评价 qPCR 实验和发表文章时所必需的实验信息提出了最低限度的标准，其中包括一个向出版者初次提交文章时所要附带的清单。通过提供相关的实验条件和分析特性，评阅人员可以更好的评估研究者实验方案的有效性。同时应该充分披露所用的试剂、序列和分析方法，以保证其他研究者能够重现实验结果。MIQE 细节可通过缩写或在线补充的形式进行发表。

总结 通过指南可以更好进行实验操作，并得到更可靠和解释更明确的 qPCR 实验结果。

Arabian

Clinical Chemistry 55:4
611–622 (2009)

Special Report

MIQE إرشادات
الحد الأدنى من المعلومات لنشر تجارب تفاعل البلمره المتسلسل الكمي (في الوقت الحقيقي) (RT-qPCR)
(الوقت الحقيقي لتفاعل البلمرة المتسلسل)

Stephen A. Bustin,[1*] Vladimir Benes,[2] Jeremy A. Garson,[3,4] Jan Hellemans,[5] Jim Huggett,[6] Mikael Kubista,[7,8] Reinhold Mueller,[9] Tania Nolan,[10] Michael W. Pfaffl,[11] Gregory L. Shipley,[12] Jo Vandesompele,[5] and Carl T. Wittwer[13,14]

يستطيع المراجعون تقويم صحة البروتوكولات المستخدمة. كما أن الكشف الكامل لجميع مواد المحاليل و التسلسل الحمضي، و أساليب التحاليل هي ضرورية لتمكين باحثين آخرين لإنتاج نتائج جديدة. وينبغي نشر تفاصيل MIQE إما في شكل اختصار أو عبر الخط المباشر للإنترنت.

الموجز: إنّ إتباع هذه الإرشادات سيشجع على ممارسات تجريبية أفضل، ويسمح بتوفر نتائج موثقة لا لبس فيها حول الوقت الحقيقي الكمي لتفاعل البلمرة المتسلسل.

الخلفية: يوجد حاليا عدم توافق في الآراء حول أفضل السبل الموجودة لتنفيذ وتفسير الوقت الحقيقي لتفاعل البلمرة المتسلسل (qPCR). تتفاقم المشكلة بسبب النقص في التفاصيل الاختبارية في العديد من المنشورات، مما يعوق قدرة القارئ على التقييم الدقيق لجودة النتائج المقدمة أو لإعادة التجارب.

المحتوى: يستهدف الحد الأدنى من المعلومات حول نشر إرشادات تجارب الوقت الحقيقي الكمي لتفاعل البلمرة المتسلسل (MIQE) دقة النتائج للمساعدة على ضمان

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Japanese

Clinical Chemistry 55:4
611–622 (2009)

Special Report

MIQE ガイドライン:
定量的リアルタイム PCR
実験の公表に必要な最低限の情報

Stephen A. Bustin,[1*] Vladimir Benes,[2] Jeremy A. Garson,[3,4] Jan Hellemans,[5] Jim Huggett,[6] Mikael Kubista,[7,8] Reinhold Mueller,[9] Tania Nolan,[10] Michael W. Pfaffl,[11] Gregory L. Shipley,[12] Jo Vandesompele,[5] and Carl T. Wittwer[13,14]

背景：現在、定量的リアルタイムPCR（qPCR）実験の最適な実施方法および解釈方法について、統一された基準がない。問題は深刻化しており、多くの文献では実験の詳細が不十分で、結果のクオリティの批評や実験の再現を妨げている。

要約：MIQEガイドラインに従うことによって、実験のよりよい慣例が推進され、qPCRの結果をより確実かつ明確に解釈することが可能となる。

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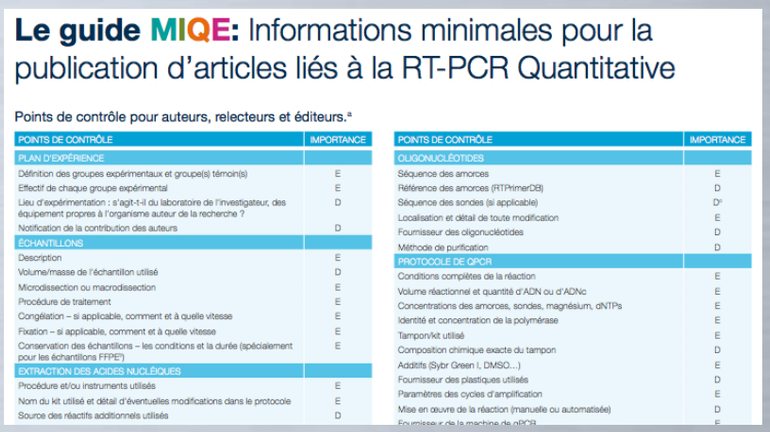
French



Le guide MIQE: Informations minimales pour la publication d'articles liés à la RT-PCR Quantitative

Points de contrôle pour auteurs, relecteurs et éditeurs.[a]

POINTS DE CONTRÔLE	IMPORTANCE
PLAN D'EXPÉRIENCE	
Définition des groupes expérimentaux et groupe(s) témoin(s)	E
Effectif de chaque groupe expérimental	E
Lieu d'expérimentation : s'agit-t-il du laboratoire de l'investigateur, des équipement propres à l'organisme auteur de la recherche ?	D
Notification de la contribution des auteurs	D
ÉCHANTILLONS	
Description	E
Volume/masse de l'échantillon utilisé	D
Microdissection ou macrodissection	E
Procédure de traitement	E
Congélation – si applicable, comment et à quelle vitesse	E
Fixation – si applicable, comment et à quelle vitesse	E
Conservation des échantillons – les conditions et la durée (spécialement pour les échantillons FFPE[b])	E
EXTRACTION DES ACIDES NUCLÉIQUES	
Procédure et/ou instruments utilisés	E
Nom du kit utilisé et détail d'éventuelles modifications dans le protocole	E
Source des réactifs additionnels utilisés	D

POINTS DE CONTRÔLE	IMPORTANCE
OLIGONUCLÉOTIDES	
Séquence des amorces	E
Référence des amorces (RTPrimerDB)	D
Séquence des sondes (si applicable)	D[c]
Localisation et détail de toute modification	E
Fournisseur des oligonucléotides	D
Méthode de purification	D
PROTOCOLE DE QPCR	
Conditions complètes de la réaction	E
Volume réactionnel et quantité d'ADN ou d'ADNc	E
Concentrations des amorces, sondes, magnésium, dNTPs	E
Identité et concentration de la polymérase	E
Tampon/kit utilisé	E
Composition chimique exacte du tampon	D
Additifs (Sybr Green I, DMSO…)	E
Fournisseur des plastiques utilisés	D
Paramètres des cycles d'amplification	E
Mise en œuvre de la réaction (manuelle ou automatisée)	D
Fournisseur de la machine de qPCR	E

Chapter 22

MIQE qPCR and dPCR online applications

Afif M. Abdel Nour

Get help from a special team of experts in qPCR while on the move. MIQE - qPCR helps you in reviewing scientific works and checking your own experiments, when qPCR is involved. Check your project's compliancy to MIQE in minutes, have all required references in hands, and follow qPCR events and news. Add to that, iCloud features are now included, so you can keep working on all your devices with the same data.

· The qPCR turns digital, so does this app. All new checklists, references dedicated to ddPCR are included!
· Now MIQE qPCR app is even more interactive. For the first time ever, checklists are optimized in real time: you can reach 100% for every project if you are MIQE compliant.
· Checklists are specific for each project type: just click on the kind of nucleic acid you are working with, the checklists adapts instantly by removing unnecessary items (Reverse transcription items are not relevant if working only on DNA for instance).
· Moreover, some items may not apply to your specific experiments. You can now remove them and have the most accurate MIQE compliancy.
· References have been updated, so you can keep in touch with latest MIQE related literature, symposium updates and more.
· Last but not least: Export is available. You can archive the state of your project whenever you want, share it with your colleagues, and store it.

Please note that Android and HTML5 apps are also available.

Chapter 23

Credits and Imprint

Afif M. Abdel Nour

& Michael Pfaffl

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Termes connexes du glossaire

Faire glisser ici les termes connexes

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