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Guest Editor's Introduction

The ongoing evolution of qPCR

The polymerase chain reaction (PCR) is usually described as a simple, sensitive and rapid technique that uses oligonucleotide primers, dNTPs and a heat stable Taq polymerase to amplify DNA. It was invented by Mullis and co-workers [1,2] in the early eighties, who were awarded the 1993 Nobel Prize for chemistry for this discovery. With the discovery of real-time PCR in the nineties the method took an important hurdle towards becoming "fully quantitative" [3]. The addition of an initial reverse transcription (RT) step produced the complementary RT-PCR, a powerful means of amplifying any type of RNA [4,5]. Today quantitative PCR (qPCR) is widely used in research and diagnostics, with numerous scientists contributing to the pre-eminence of PCR in a huge range of DNA-, RNA- (coding and non-coding) or protein- (immuno- or proximity ligation assay qPCR) based applications. Soon the PCR was regarded as the "gold standard" in the quantitative analysis of nucleic acid, because of its high sensitivity, good reproducibility, broad dynamic quantification range, easy use and reasonable good value for money [6–8].

qPCR has substantial advantages in quantifying low target copy numbers from limited amounts of tissue or identifying minor changes in mRNA or microRNA expression levels in samples with low RNA concentrations or from single cells analysis [9–11]. The extensive potential to quantify nucleic acids in any kind of biological matrix has kept qPCR at the forefront of extensive research efforts aimed at developing new or improved applications. But are qPCR and its associated quantification workflow really as simple as we assume?

It is essential to have a comprehensive understanding of the underlying basic principles, error sources and general problems inherent with qPCR and RT-qPCR. This rapidly reveals the urgent need to promote efforts towards more reproducible, sensitive, truly quantitative and, ultimately, more biologically valid experimental approaches. Therefore, the challenge is to develop assays that meet current analytical requirements and anticipate new problems, for example in novel biological matrices or for higher throughput applications. Unfortunately, we are far from having developed optimal workflows, the highest sensitivity, the best RNA integrity metrics or the ultimate real-time cycler, all of which are indispensable for optimal PCR amplification and authentic results. The qPCR research community still aims to improve and evolve, which brings to the topic of this PCR special issue – *The ongoing evolution of qPCR*.

In this issue we want to focus on some selected application fields which have been identified as indispensable for research and diagnostics:

Standardisation – Why do we need more standardisation and therefore the MIQE (*m*inimum *i*nformation for publication of *q*uantitative real-time PCR *e*xperiments) guidelines? Following these guidelines will encourage better experimental practice, allowing more reliable and unequivocal interpretation of quantitative PCR results. As we continue to improve our workflow to achieve the best and, it is hoped, the most valid results, the key message is that quality assurance and quality control are essential throughout the entire RT-qPCR workflow, from experimental design to statistical data analysis and reporting. The first paper in this issue pinpoint these key components, will help you identify the sources of errors and provide guidance towards which experimental design might be best suited to your study. Since meaningful conclusions can only derive from consistent and accurate quantification results, increased reliability of research will help ensure the integrity of the scientific literature.

MicroRNA – A second focus is on the valid quantification of microRNA. I cannot over-emphasise the importance of microRNA in the regulation and cellular turnover of the transcriptome. MicroRNAs are small non-coding RNAs (~20–22 bases) and play an important role in gene regulatory networks by binding to and repressing the activity of specific target gene messages. Within the previous decade numerous papers have been published and a range of microRNA applications has been generated. Herein we want to focus on the quality control of microRNAs in numerous tissues and to give an overview of new quantitative assays using qPCR.

High resolution melting (HRM) analysis – HRM is a relatively new application for genotyping and variant scanning after a successful PCR reaction. It can also be used to scan for rare sequence variants in large genes with multiple exons, which are described herein. HRM assay design, optimization, performance considerations and new analysis software based on cluster analysis are presented. The new HRM cluster algorithm provides a sensitive and specific auto-calling of genotypes from melting data allowing a more sensitive resolution of genetic differences.

Copy number variations (CNV) – Copy number changes are known to be involved in numerous human genetic disorders. Presented papers describe qPCR-based copy number screening methods that may serve as the "gold standard" for targeted screening of the relevant disease genes. All relevant information for a successfully implementation of qPCR in copy number analysis in a high throughput digital PCR is included. Furthermore, recommendations for appropriate copy number calculation and objective result interpretation is also addressed.

Single-cell qPCR and circulating tumour cells – Single-cell gene expression profiling is rarely undertaken, in part due to a lack of understanding of single-cell biology and the underlying high cell-

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to-cell variability. However, as the relevant paper shows, qPCR-based single-cell gene expression profiling can be a powerful tool for achieving a better understanding of molecular mechanism at the level of a single cell. In addition, the analysis in circulating tumour cells (CTCs) is described. CTCs can be released from the primary tumour into the bloodstream and may colonize distant organs giving rise to metastasis. The qPCR-based analysis of individual cells opens up new avenues for molecular biologists and for early cancer diagnostics. Presented papers describe comprehensively which considerations one has to take to avoid false conclusions during data analysis and interpretation of single-cell expression profiling data. Moreover, the focus is on the relevance of the clinical diagnostics generated so far and based on the CTCs analysis in malignancy.

Circulating nucleic acids (CNA) – Recent studies have indicated that microRNAs circulate in a stable, cell-free form in the bloodstream. The expression pattern of specific microRNAs in plasma can be used as a diagnostics tool and may serve as cancer biomarkers. Quantitative measurement of circulating microRNAs as biomarkers is associated with some special challenges, which are discussed, including those related to sample preparation, microRNA extraction and stabilisation, experimental design and data analysis. Furthermore recent reports on the importance of CNA in the intercellular exchange of genetic information between eukaryotic cells are reviewed.

Post-qPCR data analysis – In research and in clinical diagnostics enormous amounts of expression data based on quantification cycles (Cq) are created. Accurate and straightforward mathematical and statistical analysis of qPCR data and the related data management of these growing data sets have become major hurdles to effective implementation. The 96-well and 384-well applications are standard formats in research, but in the near future high throughput applications with more than 1000 PCR spots will generate huge amounts of data. Various qPCR data sets need to be grouped, standardized, normalized and documented by intelligent software applications. In the presented papers the main challenges and new solutions in mathematical and statistical Cq data analysis are presented. The so-called qPCR bio-informatics and bio-statistics field is highly variable, because a range of data processing procedures have been adopted; these are based on differing algorithms for performing background corrections, threshold settings, Cq determination or RNA expression normalisation. Herein we present statistical approaches based on multivariate analysis of the fluorescence amplification response data generated. The amplification trajectory is fitted with suitable models to analyse PCR efficiency and to establish a qPCR quality control procedure depends on a reference set.

Conclusion – The last two decades have been characterised by important methodological advances that have made qPCR more sensitive, less variable and therefore more valid and reliable. Most advances were implemented in the PCR method itself, but pre-PCR steps like sampling, nucleic acid stabilisation and reverse transcription are still highly variable and introduce lots of error in the quantification procedure. Appling intelligent post-PCR data analysis can partly circumvent these problems and "normalize out the introduced error", but there is still a general clamour for the most stable references, the most appropriate normalisation strategies or robust algorithms to calculate the PCR efficiency for later correction. Clearly, we are still halfway in terms on the entire quantification work flow!

The developed MIQE guidelines will help to improve faster in future experiments, but people really have to apply these instructions to get more valid and "true" quantification results. For the future the presented papers should help the qPCR community to improve and to perform better. But we must be aware – *the evolution of qPCR* is still continuing and will keep our researcher busy for the next decade(s)!

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