qPCR
Roundtable

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1. What, in your opinion, is currently the single largest trend with respect to qPCR technology/ methodologies?

PJD: qPCR is moving into a new realm for nucleic acid analyses in that the process is being entrusted to absolute quantification of nucleic acids. While the more simple qualitative generic amplification of nucleic acid retains future importance primarily for next generation sequencing, developments in systems biology approaches and the related requirement to integrate 'omic data has placed a great emphasis on qPCR delivering reliable quantitative data to assist the building and development of meaningful holistic models. This trend is also seen through the recent availability of HTP instrumentation formats with growing input from microfluidics to assist the handling of high numbers of small volumes.

MP: Since a few years the trend is going towards high-throughput (HT) and miniaturization. Therefore new HT cycler platforms were developed. Throughput is going up, in terms of amplified target reactions per biological sample, and reaction volume is therefore minimized down to nano-liter volumes.

Often the sample RNA has to be pre-amplified for later quantification, especially for single-cell, laser micro-dissected or limited sample studies, which leads to bias and variance problems in the resulting quantitative data.

Further problems are obvious, like MIQE compliant qPCR quality control, fluorescence readout, Cq data validity, data handling, the application of appropriate efficiency correction or normalizing strategies.

AT: The rate of discovering new genetic information increases daily as a result of next-generation sequencing (NGS). The outcome of this influx of information

is a trend towards providing answers to complex questions using qPCR. Research focus has shifted from studying single targets of interest to identifying how targets interact, and what targets interact, in a broader biological context. This type of research leads to a much stronger emphasis on the analysis of pathways or diseases, for example, by using qPCR arrays to compare a number of pathway- or disease-focused genes in one experiment. The development is also coupled with a trend towards application-based solutions with higher throughput requirements, resulting in a need for specific automation solutions. In summary, the need for more convenient technologies that simplify overall applications is inevitable.

*AT: Multimarker RNA diagnostics. However clinically approved applications are still limited I see increasing trend in use of gene expression profiling in all types of labs. This is driven by the magical promise of personalized medicine as well as by the elevated capacity of the instruments.

MK: A lot is happening in the qPCR and molecular diagnostic field. But the largest trend currently in my opinion is quality control and standardization. As shown by Tichopad et al., (Clinical Chemistry 55:10 (2009); doi:10.1373/clinchem.2009.126201) most confounding variation in a typical experiment comes from the pre-analytical step rather than the PCR. Much of the confounding variation can be removed using appropriate controls, references and tools. In Europe the SPIDIA (www.spidia.eu) project aims to standardize the preanalytical process and in US CLSI (www.clsi.org) is giving increasing attention to qPCR based testing. Several companies are responding to this trend with tools, heat labile UNG and dsDNases, RNA quality assays, instrument calibration, RNA integrity testing, and quality control software.

FM: The current single largest trend is that of using platforms in which PCR is performed in parallel in multiple individual ultra-low volume chambers / aliquots / droplets. This has many implications including the physical size of the hardware, reducing reagent requirements, facilitating high throughput analyses, the streamlined acquisition of digitized data etc as well as the type of data that can be obtained. This trend has resulted in a huge decrease in the cost per data point, although the cost of the platform can mitigate this advantage. These platforms are in many cases ideal for digital PCR and therefore have significantly raised the profile of this simple and powerful technique.

2. How, in your mind, has the landscape of qPCR shifted from when the technique was first introduced to how it is being implemented today?

PJD: The arrival of qPCR marked the beginning of the end for the 'semi-quantitative' era of gene measurement. Reproducible and meaningful quantification remains elusive and far from routine. Standard procedures are being implemented to address nucleic acid measurement, and today the main caveat for gene quantification is not the analytical method (qPCR), but inconsistencies related to sample handling, and ill-definition of the sample per se.

MP: In traditional PCR we started with pure "qualitative measurements", "YES or NO" was the answer from end-point analysis and block PCR. Applying real-time PCR for the first years we moved over to the "semi-quantitative measurements" which were based on highly variable standard curve measurements and inappropriate standard material, which gave invalid quantitative results. Nowadays we reached the level of "fully quantitative and validated results" applying validated low variable assays on reproducible cycler platforms. The final goal is to reach highly standardized RT-qPCR workflow which follows the MIQE guidelines. On the basis of the generated data material from massive parallel qPCR array or HT experiments we are able to be more exploratory. The development of dedicated expression pattern or validated biomarkers, in combination with biostatistical tools like principal component analysis or cluster algorithms, is reached.

AT: qPCR has become a standard technique similar to end-point PCR — both are established in almost every research lab. Originally, qPCR was used to quantify at "real time" the amount of DNA or RNA targets in a specific sample. However, the technique has now also evolved for other, new applications like genotyping, mutation detection, multiplex PCR, and high resolution melting (HRM). Furthermore, complex research questions can now be investigated in much more detail with the coupling of various techniques like HRM and Pyrosequencing. Due to the dissemination of qPCR into new application areas such as food testing, forensics, molecular testing, and personalized healthcare, the requirement for the global standardization of techniques has become even more crucial in order to deliver data that is comparable, even when it has been produced by different laboratories.

*AT: The capacity of tests or experiments conducted has increased significantly, moving from a single assay to multiple assays run in parallel with large number of genes and samples. This goes hand in hand with single cell transcriptomics, supporting complex study of tissue heterogeneity on large set of individual cells. Generally, the qPCR in its biochemical principle reached its technology plateau and it is the accompanying technologies focusing at the pre-analytics, sample handling, and data analysis those upgrade substantially since recently.

MK: When the technique was new the qPCR analysis was the challenge. There were many issues with the early instruments and it was a challenge designing good assays. Today several companies offer highly specific and sensitive off-the-shelf assays and even optimized panels of assays for which user-friendly readers and wizard are available for smooth analysis. Instead challenge is to design experiments such that maximum biological information can be extracted for lowest cost.

3. What country/region, in your opinion, is currently leading the way in qPCR technology/methodology and why?

PJD: Possibly because of the desire to implement a recognized qPCR related standard operating procedure, a number of mainly EU labs have produced the Minimum Information for Publication of Quantitative Real-Time PCR Experiments: the MIQE Guidelines (http://www.gene-quantification.de/miqe.html). I would therefore suggest that for the present the EU should be credited with this accolade.

MP: The US is driving the platform, HT and miniaturization idea, which is market orientated, whereas the EU is more in data analysis and quantification algorithms, which is more academic orientated. Concerning the quality and standardization parameters, I see the innovations, ideas and powers more in the European research labs. No innovations are coming from the entire Asian field – still copy-and-paste mentality.

AT: Today, qPCR is a widespread technique that is used universally in all research and molecular testing labs around the globe. Innovations come from all over the world, including all major regions like Europe, North America, and Asia. Fast-paced developments can be particularly observed in Asia, and are reflected by the rapidly increasing number of patent applications related to qPCR.

*AT: Europe, in particular due to strong competence centers in Gothenburg, Munich, London, Prague and Gent. This manifest by high quality and abundance of events organized predominantly in Europe. As a European I may be biased though.

MK: US is leading technology development, reflected by high throughput qPCR instruments and digital PCR platforms, although in the molecular diagnostic field two out of the three leaders are European. Europe is ahead in methodology development as reflected by customized solutions, qPCR analysis software, and standardization (www.spidia.eu).

FM: There is no doubt in my mind that North America is leading the charge on this one. Although there is interesting work emerging from Europe and Asia the main innovations appear to be coming from North America. The "why" is more difficult to answer and in fact the geographical focus of innovation may well change over the next few years. The current status is probably a reflection of history, scientific infrastructure in terms of engineering and PCR expertise and the availability of venture capital.

4. If things progress as they have during the past five years, what can we expect in the next five years, with respect to qPCR technology/methodology?

PJD: I hope that the ability to quantify nucleic acids reaches a point when the unit definition of nucleic acid copy number is extended to take into account the cell (or volume of biological fluid) from which the nucleic acid was extracted. This approach will enable a unit definition of nucleic acid expression/abundance to utilize the cell as the common denominator which will greatly assist the ability to reproduce studies and permit a physiological relevance to qPCR-based studies. The enabling technologies offered through miniaturization and informatics are providing the necessary throughput and analysis respectively. So in terms of knowhow, these will pretty much be delivered in the next five years. It may take an additional five years hence to see further applications being derived and I suggest that these will be far more translational than available presently as the complexity of clinical matter (in terms of functional activity of somatic fluids, structural architecture, and cellular heterogeneity) will become addressable via qPCR. This will enable the distribution of cell activities and microenvironments associated with cell populations to be apparent, which in turn will help to ascertain the critical number of cells that need to be sampled to permit understanding of the biological situation. The prediction is therefore for an important and enabling move towards personalized healthcare as the differences between individuals will be characterized as a function of cellular activities and interactivities. In many respects the main change could be perceived as a seamless union between sample preparation with qPCR.

MP: Affordable and reliable chemistry and qPCR arrays, pre-validated for any physiological pathway or any application (mRNA, microRNA, SNP or epigenetic studies). They should be easy to use, validated on different biological matrices and should be robust against inhibitors. These should be run on any platform and be suitable to any analysis software or data analysis algorithm.

AT: We expect that PCR-based molecular diagnostics will expand significantly as this is a technique that can deliver much faster results than current methods and will further improve patient management. It also has great potential to become an important tool for companion diagnostics to support personalized healthcare. For example, qPCR is an ideal technique to identify specific mutations in cancer, enabling therapy for a particular mutation to be tailored for the patient.

*AT: Besides false discovery noise generated by statistically unsupervised use of the technology's capacity, I believe that manufacturers will continue highlighting technical benchmarks of their instruments while ignoring the importance of pre-analytics and experiment design. Most probably, the peripheral accompanying technologies such as single cell pre-processing, robotics and data analysis will make a significant move. I also believe that there is substantial increase in awareness for quality of published data of which the MIQE is the most tangible evidence. Researchers realized that it is the overall good control of all steps preceding and following the thermal cycling rather than the qPCR alone those have fundamental influence on the data quality. Standardization is certainly of key importance to grant a trust in the results. Briefly, the methodology will dominate the technology.

MK: Technology and methodology is developing towards lower complexity and higher throughput. In particular single cell studies are expected to grow in importance. This requires development in sample disintegration, cell sorting and enrichment, where we see exciting development within, for example, the circulating tumor cell cancer diagnostic field (www.adnagen.com). Novel reagents that allow extraction, RT and qPCR of single cells without material loss due to washing are being developed including methods to pre-amplify the material allowing profiling of even minute amounts, and then analysis in high throughput qPCR platforms. On methodology we see development of new applications including analysis of non-coding RNAs, and high resolution melting as low cost alternative for sequence and methylation analysis.

FM: Firstly, I anticipate that the profile, usage and range of applications of digital PCR will increase as users have an increased understanding of the simplicity and strengths of the protocol, as well as ready access to appropriate platforms.

Secondly, the improved integration of systems, for example to facilitate seamless/ stream-lined extraction of nucleic acids, high-throughput PCR-based analyses and/or next-generation sequencing of the amplicons.

Thirdly, there has been huge interest in the ability to interrogate gene expression at the single cell level. There is likely to be methodological refinement and innovative technology in this area.

5. In your opinion, what research field is benefitting most from the implementation of qPCR and why?

PJD: Quantification of nucleic acids has been sought for several decades, but other than a few exceptions we are not equipped to deal with assessing the full impact of nucleic acid copy numbers. The molecular biological sciences have to a greater extent made excellent use of qualitative analyses, and probably the cancer biologist should be credited with the research field that has best made use of quantitative data as this discipline has largely compared differences between normal and disease sample/patient cohorts. These studies have moved ahead of late with a desire to affix a mechanistic and functional comprehension to dysregulation through data integration and adoption of holistic modeling.

MP: Exceptionally all research fields are benefitting! Do you know any lab working in the wide field of molecular biology applications which has no real-time PCR platform, or at least access to one in the neighbor lab or core facility?

AT: The dissemination of qPCR throughout multiple labs

worldwide will surely require further efforts towards standardization. It is, therefore, not surprising that around 40% of scientists have already heard about the MIQE guidelines and are making an effort to be compliant with them.

*AT: Expressed as a grade of trust in a reaction conducted, medical diagnostics certainly draws the largest benefit as it is based on robust and validated routine. This facilitates valid and reproducible biological conclusions. Experimental use is often subject to false discovery and missing validation, the informative value per reaction is low.

MK: All research fields that benefit from the characterization of cell phenotypes, identifying cell types and cell subtypes; with single cell qPCR novel cell types and cell types will be discovered and characterized, as demonstrated by Stahlberg (Nucleic Acids Research, 2010, 1–12, doi:10.1093/nar/gkq1182). This will be particularly important in stem cell, cancer, fertility, and developmental research to name few areas.

FM: The delivery of personalized medicine and companion diagnostics is a particularly "hot" topic at present. PCR is a mature technique that has specific qualities – robustness, precision and significant experience in its application to clinical medicine – that make it very attractive in this field. PCR/qPCR is already contributing significantly to the delivery of personalized medicine and there is every likelihood that it will remain central to the goal of achieving economically viable individualized 21st century medicine

6. What recent improvements have been made to qPCR technology/methodology to ensure it keeps pace with industry?

PJD: Which industry as qPCR relates to many? The PCR usually finds itself as the pace-maker to which other technologies compete. The high quality of reagents, though little discussed now a day, facilitates highly reproducible and sensitive qPCR studies.

AT: We have responded to the need to facilitate complex analyses with a number of innovations. Master mixes that enable faster cycling and that can be universally used on any cycler are a prerequisite, especially for core facilities offering services to multiple departments. Higher throughput formats, such as FastLane allow direct qPCR analysis of cultured cells, and screening tools, such as PCR arrays, offer new application-based technologies for the analysis of a larger set of genes relevant to the pathway or disease of interest. Multiplexing capabilities and master mixes delivered with internal controls improve, and thereby increase, throughput and process security (e.g., in the routine quality control of vaccine production).

*AT: High throughput and miniaturization

MK: Industry requires standardization, to ensure that the same results are obtained when analyzing a sample independently of the kits, reagents and assays used. This is major challenge because of the very large costs involved and the very large number of standards needed, assuming a standard is needed for every target. The European SPIDIA project (www.spidia.eu) is one step in that direction and standardization was also in focus of the recent qPCR symposium held in Prague. The trend will continue at next year's meeting in Korea (www.qpcrsymposium.eu). The field has also started receiving attention from standardizing bodies.

7. The term "next generation" is seemingly being applied to everything these days. What in your opinion is the "next generation" of qPCR technology and methodologies?

PJD: A greater presence of automation is certain to abound. An enhanced union between sample handling, extraction and qPCR will be best achieved via an integrated robotic platform. These already exist, however the difference being suggested is that the next generation will respect the sample to permit, for example, intact cells to be removed, analyzed and images to be produced that resemble nucleic acid content and associated activities. This approach will facilitate virtual tissues or microenvironments to be constructed from which function can be accurately derived.

MP: The "next generation qPCR" is ultra-high throughput used in any droplet qPCR application. There digital-PCR and highly parallel quantitative assays will be possible, in short time, high accuracy and with low variability.

*AT: Quick portable multimarker qPCR devices with predefined algorithms for diagnostics use at point of care with automated sample pre-analytics. Avoiding greatly uncontrolled shipment of samples from doctor to lab will improve sensitivity and specificity and speed-up decisions in personalized approach to patients. A great deal of commercial synergy between drug use and diagnostics may propel introduction of these tests, directly or indirectly sponsored by drug manufacturers.

MK: I don't think we do the kind of leaps in qPCR development to motivate calling any improvements next generation. But the high throughput platforms are new generation in the sense that they open for profiling experiments that have not been feasible before. The digital PCR platforms are also a next generation allowing for greater accuracy and having specificity advantages in certain applications. Also the less priced small lab bench instruments running few samples only that are becoming available from several manufacturers represent a new generation qPCR instruments affordable and suitable for the small laboratory, the lightcycler nano, to mention a few.

FM: I think it is unfortunate that "next generation" is now applied by some to qPCR. It is a meaningless term in this context which does not add to the understanding of what a product/platform can deliver. Although an obvious attempt to jump on the bandwagon of "next-generation sequencing", I'm not clear that this will work. I believe microdroplet-based PCR technologies have significant advantages and I look forward to future technological developments in these platforms. Finally efficient approaches to multiplexing hundreds of loci on individual diagnostic biopsy samples need to be found.

8. Has there been a noticeable impact of qPCR on gene expression analysis?

AT: Although qPCR was initially used as a validation tool for selected targets identified by microarrays, it has replaced microarrays and is the standard technology used for gene expression analysis due to higher reproducibility, sensitivity, and the requirement for less template. As a result of the availability of genome-wide assays for many species, gene expression analysis can be performed on almost any target, including in a pathway- and disease-related context.

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