EXPERT REVIEWS

Developments in real-time PCR research and molecular diagnostics

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Developments in Real-Time PCR Research and Molecular Diagnostics

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This meeting was designed to highlight the wide range of new methods, instruments and applications that underlie the popularity of quantitative real-time PCR technology in all areas of life science research, as well as in clinical diagnostics. It provided a fascinating snapshot of current trends and novel approaches, as well as important issues concerning assay design, optimization and quality control issues.

Mikael Kubista (TATAA Biocentre, Göteborg, Sweden) opened the meeting with a wide-ranging overview of the latest real-time quantitative PCR (qPCR) ideas. He emphasized the analytical problems associated with sample complexity and tissue heterogeneity, the need for appropriate assay design, how variability of RNA stability affects results and how it is the correlation between mRNAs, rather than mRNA expression levels themselves, that defines cellular behavior and identifies different cell types. In terms of future challenges, he listed the need for stringent and robust standard operating procedure development, advized on the use of exploratory pilot experiments to test every step of assay performance and identify experimental variability, and strongly advocated multimodal data analysis methods for result interpretation. He concluded by pointing to the exciting prospects ahead for qPCR in diagnostic and theranostic applications.

Vladimir Benes (EMBL, Heidelberg, Germany) described miQPCR, a new method for parallel profiling of primary (pri-), precursor (pre-) and mature miRNAs by qPCR. miRNAs are small noncoding RNAs that comprise approximately 1% of transcripts in plants and animals and regulate gene expression post-transcriptionally by specific base-pairing to their target mRNAs. They participate in the regulation of almost every cellular process and their dysregulation is linked to cancer and disease development. Their analysis, let alone quantification, is difficult as they exist as: pri-miRNAs that can span hundreds or thousands of nucleotides in length and contain more than one miRNA; pre-miRNAs of approximately 70 nucleotides derived from the pri-miRNAs and mature miRNAs. This is exacerbated by the short length of mature miRNAs, their variable T_ms that range from 40 to 80°C and the presence of related family members. Benes described the development of an improved, more flexible system for the accurate quantification of miRNAs that employs a one-step reverse transcription of all miRNAs contained in a sample, makes use of a simple and flexible primer design that allows T_m adjustment of miRNA-specific primers and has high specificity in detecting and discriminating between closely related sequences. The method involves the dephosphorylation of a total RNA preparation containing small RNA, ligation of its 3′-end with a single-stranded T4 RNA ligase to the 5′-end of a phosphorylated specific RNA/DNA adaptor (miLINKER), reverse transcription of the ligated small RNA using a universal primer, removal of all single-stranded RNA and then cDNA synthesis using an upstream miRNA-specific primer. Both universal and miRNA-specific primers are then used during the qPCR for the detection of the selected amplicons. Benes described this technique as an open, adaptable and cost-effective procedure for miRNA expression profiling by qPCR.

Pavel Neužil (Korea Institute of Science and Technology, South Korea) has a long-standing interest in replacing conventional,

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laboratory-based PCR equipment with point-of-care microPCR devices, where the need is for a rapid response, rather than the detection of numerous targets. This has now resulted in the development of a simple device with built-in thermal management, a fluorescence-based detection system and a single chip controller with touch-screen graphic display. qPCR is carried out in virtual reaction chambers of water-based samples encapsulated in oil droplets on a silicon-etched chip covered with a standard microscope cover slip. Each chip contains a number of reaction chambers for genes of interest and control genes, and each reaction chamber is heated using miniature electrical elements. Each assay is carried out in tiny reaction volumes, typically 100 nl of sample within 1 µl of oil, on disposable glass slides that minimize contamination, with rapid cycle times, typically heating from room temperature to 150°C in 0.2 s resulting in cycling times of less than 10 min and a low power requirement that allows it to run on battery power. The chip sits in an optical detection device about the size of a portable DVD player, indeed the optical detection module is similar to the optical detection system of a DVD player and uses a blue LED excitation source, fluorescein isothiocyanate (FITC) filter set and photodiode with amplifier. The latest instruments can detect two colors, have increased detection sensitivity and can be powered from a USB source. The total cost is approximately US$120. Neužil referred to one potential problem, common with all PCR-based assays, relating to sample preparation and the need to ensure absence of inhibitors.

Anders Lönneborg (DiaGenic, Oslo, Norway) described two qPCR-based tests that are based on two notions: diseases such as cancer or Alzheimer's disease (AD) have a systemic impact, leaving unique signatures in nondiseased parts of the body, including the circulatory system; and that these responses can be detected by measuring amounts of RNA for specific genes from peripheral blood. Both qPCR tests use peripheral blood samples collected in PAXgene™ (PreAnalytiX GmbH) tubes and are subjected to a number of quality control checks; they rely on the ability of the assay to detect disease-associated gene-expression signatures from sets of informative genes. The assays utilize Life Technologies' 96-gene microfluidic card format run on an ABI 7900HT system. Importantly, in validation studies, external factors, such as variability in blood collection and testing methods, common comorbidities, interference from other common medications, contraceptive use, menstrual cycle and age, all had no bearing on the accuracy of either test.

The first test (BCtect) targets breast cancer, which remains the main cause of cancer death among women. Mammography and histopathology/cytology are sensitive screening and diagnostic tools; however mammography is less sensitive in younger women, women with dense breasts and for cases of lobular carcinoma, which often lack micro-calcifications. It targets mRNA expression, but differs from other prognostic assays that rely on expression signatures: its markers are not obtained from tumor tissue, instead this assay is based on the detection of blood-based mRNA transcripts that are altered in response to tumor growth. Initial studies had demonstrated that peripheral blood gene expression can provide information to distinguish breast cancer patients from healthy age-matched controls. Using blood from 140 subjects, a number of whole genome studies using various microarray platforms identified 700 informative mRNA transcripts that distinguished breast cancer patients from age-matched controls with a high accuracy of 81% (sensitivity 82% and specificity 79%). To identify the optimal gene signature, 384 of the most informative gene transcripts were selected, validated using qPCR and narrowed down to a gene-expression signature of 96 genes, including several control genes using a new cohort of 123 patients. The BCtect assay was validated using a multicenter study that used approximately 550 blood samples from five recruitment sites in Scandinavia and the USA. As with all cancers, early detection of a growing breast tumor is of key importance for patient survival. The BCtect assay, which is CE-marked, is as effective at detecting early stage breast cancer (stage 0 and 1) in premenopausal women as it is in postmenopausal women, successfully detects lobular carcinomas and has an overall accuracy between 75 and 85%. Results are typically available within 7 days from shipment of the sample to the central laboratory and are reported as either normal/negative or abnormal/positive. Since false-negative results can occur, the test must always be used in conjunction with the patient's clinical data and results from other tests to establish a final diagnosis. A positive test result should be followed up by an appropriate imaging modality to localize the lesion and if necessary a biopsy or fine needle aspiration to obtain a definitive diagnosis. Importantly, the BCtect test may detect cancers prior to their detection by conventional tests, as the cancer may be too small for detection by mammography, or may be hidden by dense breast tissue. Hence it is important to exclude a false-positive result by performing additional clinical examinations.

Alzheimer's disease is the main cause of dementia in the Western world and currently afflicts approximately 15 million people worldwide. As life expectancy increases, the prevalence of AD is expected to rise dramatically and it will become an even greater severe health problem in the next few decades. Intense research is ongoing to identify reliable cures or preventive measures for the disease. To help these investigations, there is an urgent need to identify reliable biomarkers that can detect the disease at the earliest possible stage. The AD Test (ADtect) aims to detect mild-to-moderate AD and is based on the same principle of detecting unusual mRNA signatures in a person's blood that indicates the presence of AD. It provides a convenient add-on test to existing clinical examination and, by detecting AD at an early stage, can help secure the most appropriate healthcare for the individual. The test has a high accuracy in patients with mild cognitive symptoms, which are the most difficult patient group to diagnose. The target mRNAs were selected following several clinical studies that used blood samples from subjects with early-stage disease and a multicenter validation study showed an accuracy level of 72%. The same considerations with respect to false-negative and -positive results as discussed with the BCtect test apply.

Roland Nagel (Applied Biosystems, CA, USA) described a novel, hot-start thermostable reverse transcriptase that promises to improve the specificity of the cDNA synthesis step by eliminating the primer-independent priming that often occurs. There

are a number of issues that are essential when considering the use of an reverse transcription (RT) enzyme: ideally it should not be very active at room temperature, as such activity results in mispriming from nonstringent primer annealing. Any hot start procedure must be well below the temperatures used for hot-start DNA-dependent polymerases, as RNA is degraded at temperatures above 65°C. Reactivation should also be rapid and efficient. Nagel described the properties of a chemically modified thermostable RT enzyme that fulfils all these requirements: it is fully deactivated at RT, high levels of activity are recovered following activation, it can be activated at temperatures that are compatible with RNA integrity and chemical modification is stable for at least 4 months. When compared with RT reactions carried out with nonhot-start enzymes, there appears to be substantially more specificity of the RT, with the RT able to distinguish between RNA sequences with a single base mismatch.

The final session of the meeting included a number of talks discussing some of the problems, and proposing solutions, associated with qPCR results. Alexandra Vamvakidou-Thomas (Integromics, Madrid, Spain) discussed some of the challenges associated with obtaining reliable results, with a particular emphasis on data analysis. Her key points related to the need for replicates, appropriate sample size and quality control. She emphasized the need to set up regular check points during sample preparation, processing and data analysis to reduce variance and ensure each time-reproducible results. She concluded that data analysis requires a strict workflow if results are to be reliable and reproducible. Ales Tichopad (Technical University Munich, Germany) discussed the design and optimization of RT-qPCR experiments. He described the estimation of the errors that are introduced into mRNA measurement by sampling, nucleic acid extraction, RT and the qPCR step itself. Echoing Kubista's talk, he showed how in experiments utilizing single cells or solid tissue biopsies the inter-subject differences get easily confounded by sample heterogeneity. By contrast, sampling noise experiments involving blood and cell cultures is less pronounced, whereas noise from the RT and qPCR steps contributes significantly to the overall error. He emphasized the need for biological replicates when working with solid tissue, cell cultures and single cells, whereas RT replicates are essential when working with blood. Stephen Bustin (Queen Mary University of London, UK) described the thinking behind MIQE, the minimum information for the publication of qPCR experiments. These guidelines were conceived to encourage more comprehensive communication of experimental detail within publications utilizing qPCR technology and their incorporation of the many variables that make up the pre-assay, assay and data analysis steps, making these guidelines an ideal template for qPCR assay. MIQE is beginning to have an impact on the quality of data published, although some journals, frequently with high impact factors, continue to publish misleading and technically inadequate research papers. Bustin called for a concerted effort to implement these guidelines to allow qPCR to mature into a robust, accurate and reliable quantification technology. Finally, Michael Pfaffl (Technical University Munich) dealt with the problem of how to adapt the MIQE guidelines to miRNA expression profiling. He demonstrated that quantification of miRNA is highly dependent on the integrity of total RNA and suggested that for tissue biopsies a RNA integrity number/RNA quality indicator more than 5 is indicative of an RNA sample suitable for miRNA quantification. Pfaffl also carried out an intra- and inter-platform comparison of miRNA expression analysis and normalization techniques. He concluded that normalization using Loess, LoessM, invariant selection or generalized procrustes analysis is best for intra-platform normalization, as all four revealed highest trueness in identifying differentially expressed miRNAs. For inter-platform normalization Pfaffl recommends GeNorm-style normalization for single RT-qPCR assays, with GeoMean normalization for RT-qPCR arrays as the most reliable means of identifying differentially expressed miRNAs. However, he emphasized that appropriate controls or databases for miRNA reference genes are missing and that there is an urgent need for developing better normalization strategies for miRNA quantification.

In summary, this meeting fulfilled its aim of providing a snapshot of current thinking in the qPCR field and provided important new guidance on how to determine the actual clinical relevance of a qPCR result, but emphasized that there is still no consensus on how to report them.

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