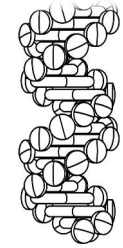

Nucleic acid-based technologies: application amplified

Cambridge Healthtech Institute's 12th Conference on Nucleic Acid-Based Technologies,
June 21–23, 2004, McLean, Virginia, USA

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In vitro diagnostics is one of the largest markets in the medical technology field, with a revenue of > US$22 billion. Nucleic acid probes tests, estimated at US$1.2 billion in 2002, are forecast to have about five times the growth rate of other products within the diagnostic market over the next 5 years (compound annual growth rate [CAGR] of 21%). Amplified methods are considered to be the primary growth driver and are projected to represent between 75 and 90% of the market in 2006. The technologies, tools, and new applications presented at this year's meeting supports these optimistic predictions. Also, the rapidly expanding market, leaving space for many players, was reflected by the participants, who were actively seeking partnerships, technological synergies, and complementary methodologies.

Keynote presentation

The conference began with a keynote presentation by Michael Zuker, who is professor in Mathematical Sciences at the Rensselaer Polytechnic Institute, NY, USA. Dr Zuker was probably not as well known to the audience as his very popular software mfold, used by thousands of scientists around the world to design PCR primers [1]. Zuker gave a explanatory lecture about the basics of oligomer folding and equilibrium thermodynamics. He started by explaining the significance of single strand secondary and tertiary structures to hybridization energies. He then described statistical thermodynamic approaches to account for all the possible structures that single strands may adopt, in order to estimate thermodynamic data and, in particular, melting temperatures. Based on this reasoning he explained how the secondary structure of probes, such as molecular beacons, may lead to higher sequence specificity. Finally, Zuker emphasized the inappropriateness of using Basic Local Alignment Search Tool (BLAST) and similar search programs to predict thermodynamic properties of oligonucleotides for serious applications.

Detection methods for quantification of gene expression

The first session was devoted to nucleic acid detection methods. Siao Ping Tsai from Genentech described the nucleic acid capture assay (NACA) for direct quantification of nucleic acids. NACA is a further development of Genentech's branched DNA (bDNA) technology, in which labeled extender oligonucleotides are partially hybridized to a mRNA target. One set of extenders captures the target by hybridizing to it with one end, and with the other end it hybridizes to a DNA oligomer immobilized on a solid support. A second set of extenders hybridizes to the target with one end and to the branched DNA amplifiers with the other. An alkaline phosphatase conjugated DNA probe then hybridizes to the branched amplifiers, generating a colorimetric response. Disadvantages of the original bDNA technology are high background and high cost, which is partially due to the complexity of the system. In NACA, the target mRNA is immobilized by direct hybridization to oligomers on a solid support. Optimum efficiency was obtained when 2′-*O*-Me-RNA was used with 3′ ethylene glycol scaffolding as immobilizers. The branched amplifier is also replaced with a stem oligonucleotide to which specific probes labeled with digoxigenin (DIG) are bound. These, in turn, are bound by alkaline phosphatase-conjugated anti-DIG antibodies. The advantage of this direct multi-capture system is its versatility, which makes it suitable for arraying.

Shane Xin from AlleLogic Biosciences presented AllGlo™, which is a novel probe technology for real-time PCR. Like Taqman® and the Molecular Beacons, AllGlo probe has two dye labels with the potential of including more dyes in future versions. However, in the case of AllGlo, the dye labels are identical and, like with LightUp™ probes, the fluorescence of the dyes increases when AllGlo binds. AllGlo can also be used as a primer, and it can be designed to be degraded by the Taq polymerase 5′ nuclease activity like Taqman, leading to fluorescence increase. The signal increase observed with AllGlo is larger than that observed with Taqman probes. The dyes used in AllGlo were not disclosed, but they were said to be available in several colors for multiplexing and SNP typing.

Mikael Kubista, from the TATAA Biocenter in Sweden, described the BEBO dyes (minor groove-binding asymmetric cyanine reporter dyes), which, like SYBR® Green become fluorescent upon binding nucleic acids, and are excellent non-specific labels in real-time PCR. The BEBO dyes have better water solubility than SYBR and they bind in the DNA minor groove, which makes them specific for double-stranded DNA, since single strands have no grooves. Kubista further presented the peptide nucleic acid (PNA)-based

LightUp probes, which also become fluorescent upon target hybridization. They make use of the ability of asymmetric cyanine dyes, such as BEBO and SYBR, to become fluorescent in the presence of DNA, and the LightUp probes are essentially sequence-specific BEBO/SYBR labels. Kubista also described experimental strategies to measure relative expression of target genes that take into account both reverse transcription yields and sample-specific PCR inhibition. The approach was illustrated with an assay for non-Hodgkin's lymphoma based on measuring the relative expression of the κ and λ variants of the immunoglobulin light chain. These data were analyzed by GenEx™ from MultiD Analyses [2], which is software developed for the selection of reference genes and for the classification of test samples, with and without training sets, characterized by the expression of several markers. Finally, Kubista presented the high-throughput real-time PCR platform that is being developed at WaferGen, CA, USA [3].

Peter Mouritzen from Exiqon, Denmark, presented the locked nucleic acid (LNA) probe library [4] for real-time PCR applications. The library exploits the greater hybridization stability of LNA compared to normal nucleotide bases. Probes eight to nine bases long and containing LNA bases are sufficient to hybridize to a target sequence at elongation temperature. Strategically designed, 90 such LNA probes are sufficient to target 37,000 (98%) of the human genome transcripts, and 90% of the Ensemble mRNA transcripts with intron-spanning assays. The success rate using the LNA probes in real-time PCR assays was 97.5%. A rat LNA probe library will be released during 2004.

After a technology workshop sponsored by BD Biosciences, Clontech, CA, USA, Bob Larsen presented the Qzyme™ assay. QZyme is based on *in vitro*-evolved catalytic DNA, so-called DNAzyme, which cleaves complementary sequence in the presence of Mg^{2+}. The DNAzyme consists of a catalytic domain flanked by two substrate recognition sequences. When it hybridizes to complementary sequence, the latter is catalytically cleaved. QZyme contains a gene-specific 5′-primer conjoined to the inactive, antisense strand of the DNAzyme, and a gene-specific 3′-primer. Also present is a DNAzyme-specific fluorogenic substrate, which is a short nucleic acid segment tagged with a fluorophore at one end and a Black Hole Quencher™ at the other. When the 5′-primer is extended by PCR and copied in the next cycle it produces the catalytic sequence, which then cleaves the fluorogenic substrate that becomes fluorescent. QZyme is readily multiplexed by varying the DNAzyme recognition sequences and the QZyme target sequences. Since everything else is identical and there are no probes that may interfere with the primers, the multiplex assays are exceedingly robust, and several triplex and quadruplex assays were presented. Also described was a new antibody-based hot-start Taq that reduces primer–dimer formation to a minimum in any real-time PCR assay.

Alternative amplification methods

The second session focused on alternatives to PCR. The first speaker was Guillermo Paez from Harvard Medical School, MA, USA, who talked about ϕ29 polymerase-based multiple-strand displacement whole genome amplification (WGA). WGA is performed with the highly processive ϕ29 DNA polymerase, which also has strand displacing activity, using random exonuclease-resistant primers. Starting from a 10 ng template ~ 45 µg of DNA was produced in this way. The amplified material was compared with non-amplified genome by sequencing and also by SNP analysis using array hybridization. Only six sequence regions, representing a maximum of 6.52 Mb, were not found to be amplified. Estimated genome coverage was 99.82%. Gene copy numbers, both deletions and amplifications, were preserved. When sequencing 0.5 million bases, the sequencing error in the WGA material was 9.5×10^{-6} compared to 7.6×10^{-6} in the non-amplified material.

Huimin Kong from New England Biolabs, MA, USA, presented helicase-dependent amplification (HDA). When using helicase to separate duplex strands, DNA is amplified isothermally. Targets of ≤ 1 kb were successfully amplified when using a processive helicase. HDA can also be combined with immunoassays for protein detection, reaching a sensitivity that is sixfold higher than with traditional ELISA. In its present form the method requires postprocessing for quantification, but a colorimetric system for real-time detection is being developed.

Larry Wangh from Brandeis University, MA, USA, presented an advanced form of asymmetric PCR called the linear-after-the-exponential (LATE)-PCR. The two PCR primers are designed with different melting temperatures, which makes it possible to control the switch from symmetric to asymmetric amplification efficiently. The system is particularly advantageous for genotyping. Wangh also presented data with a new buffer called Elixir that efficiently suppresses primer–dimer formation.

Ernest Mueller from Sigma-Aldrich presented Restorase™, which is a blend of DNA polymerase and repair enzyme for amplification of damaged DNA. Not all damages can be restored, though, but lesions are repaired with high efficiency.

Nalini Raghavachari from the National Institutes of Health (NIH), discussed the problem of obtaining a sufficient amount of RNA from blood samples for microarray analysis. The heterogenic population of blood cells and insufficient amounts of homogenous cell types make it necessary in most cases to amplify RNA for microarray analysis. When preparing platelet RNA, < 100 ng RNA is obtained from 20 ml of blood. The investigators tested the efficacy of T7 amplification on RNA from sickle cell patients. The RNA was reverse transcribed to full-length double-stranded cDNA and then transcribed *in vitro* from a promoter-primer to generate antisense RNA. A total of 50 ng of RNA was amplified in two rounds and analyzed by hybridiza-

tion to microarray. The results were compared with non-amplified RNA analysed by real-time PCR. They found that amplification does introduce a bias in the expression profile, but, nevertheless, quantitative and biologically significant data could be obtained when nanograms amounts of starting RNA were available.

Joe Don Heath from NuGEN Technologies, CA, USA, presented Ovation™, which is a new isothermal RNA linear amplification system. A DNA–RNA chimera primer with an oligo(dT) segment that hybridizes to the mRNA poly(A) tail is used for first strand cDNA synthesis. The RNA is then fragmented and a second cDNA strand is synthesized, which produces a terminal DNA–RNA heteroduplex. RNaseH and a SPIA™ DNA–RNA chimera primer are added. In a cyclic process, the terminal RNA part is degraded by the RNaseH, leaving room for new SPIA primers to bind and initiate transcription of more RNA. Four orders of magnitude dynamic range is obtained when starting from 5–100 ng of total RNA.

The key to quality – sample preparation

The second day was devoted to sample preparation. The first speaker was Mike Brownstein, Chief of Laboratory of Genetics, NIMH/NHGRI, who gave a talk on collecting and processing small tissue samples for microarray studies. They had found it almost impossible to extract good quality RNA from formalin-fixed cells and from ethanol-fixed tissues. Best results were obtained with DSP, which is a reversible crosslinker with a sulfur bridge, fixed and post stained cells, from which RNA with reasonable integrity could be extracted. They had developed an RNA amplification system based on random priming, which worked better on partially degraded mRNA samples than poly(dT)-based amplifications. Their current limit for quantitative analysis was 1–10 ng of RNA template (typically 200–2000 cells). Brownstein also showed an image of an 'empty' (unloaded) microchip, where a number of spots were seen (some being very bright) despite the fact that the chip had never been in contact with sample nucleic acids.

The next speaker was Kevin Jones from Whatman, who talked about membranes and solid-phase matrices for sample collection and preparation. Their track-etched membrane has pores of known size formed by charged particle bombardment. The pores are a few micrometers in size and the membranes can be used, for example, to remove cells by filtering. Their FTA® filter has a matrix to entrap nucleic acids for storage and purification purposes. Cells are lysed upon contact, proteins are denatured, and nucleic acids are immobilized. The nucleic acids can be stored in the FTA filter matrix for at least 1 year. The material is recovered from the filter by punching out a piece, from which the nucleic acid is purified in < 30 min for, for example, PCR analysis.

Rudolph Spangler from the Rockefeller University, NY, USA, then talked about high-throughput RNA purification for reverse transcriptase (RT)-PCR analysis. Spangler stressed the importance of inactivating RNases by salting out macromolecules, for which he recommended using ~ 50% ammonium sulfate. He also described an elegant high-throughput method for purifying RNA by using a three-phase extraction system based on an aqueous phase, a polymer, and an organic phase. He also recommended the LocusLink interactive database [5] to link disease to genetic loci.

Oliver Kreft from the Max Planck Institute, Germany, described a micro vehicle for DNA storage, separation, and PCR. On a charged colloidal particle made of dissolvable material, oppositely charged polymers are alternately deposited. After the addition of several layers, the core is dissolved, which leaves a hollow polyelectrolyte microcapsule. The core can be made of various materials; it can even be a cell. The polyelectrolyte layers can be various inorganic polymers or biopolymers, such as polysaccharides or nucleic acids and the assembly can be made such that large molecules are trapped inside the microcapsule. The microcapsule can also be filled through drying, which makes it permeable, and upon resuspension in water it is impermeable again. Examples were shown with trapped oligonucleotides, and the goal is to use the microcapsule as a container for PCR, where the reaction components can diffuse into the capsule while the target is trapped.

The next speaker was Xingwang Fang from Ambion, who spoke about RNA isolation. First, he compared traditional methods based on gel filtration and magnetic beads, showing that the bead methods have slightly better yields and higher reproducibility. Both approaches had a large dynamic range. Fang then presented a procedure based on a single lysis buffer that is added to pelleted cells, and which is compatible with both DNase treatment and RT-PCR. A number of examples of going from cells to cDNA in one step were presented. The second generation cell-to-signal lysate required neither heating nor the addition of DNase, and was compatible with RT-PCR. Under optimum conditions, 96 samples were prepared for RT-PCR in 2–5 min. The same lysate can be used for protein quantification by ELISA and immunoblotting.

To an excellent lunch sponsored by Eppendorf 5′, Lars-Erik Peters first presented a new hot-start technology called the HotMaster™. It is based on tuned competitive binding of a synthetic polymer to the Taq polymerase at lower temperatures. This makes the hot-start function reversible, reducing primer–dimer formation even at low temperatures during the PCR. Peters also demonstrated the importance of buffer on PCR efficiency and on the stability of cDNA during freezing and thawing.

Applications amplified

The afternoon session was devoted to high-throughput applications, and the first speaker was Neil Gibson from AstraZeneca. Neil talked about the application of genotyping technologies to the drug discovery and development process. He described their Laboratory Information Management System (LIMS), which was

based on Applied Biosystems (ABI) assay on demand/by design. The accuracy and reproducibility of the SNP assays were > 99.9%. Out of 341 submitted SNP sequences, 96% passed design, 80% passed suppliers testing, and 79% worked in the hands of the users. Their goal was to apply WGA to dissect the genetic component of clinical phenotypes. For WGA, 125,000–500,000 SNPs need to be tested in some 1000 samples. With their present throughput of 50,000 samples/day it would take 38 years. To make WGA feasible, throughput must reach 5 million tests per day. SNP analysis can be scaled using a generic high-throughput technology, such as that developed by Perlegen, where the genomic material is first restriction digested and then, using adaptor technology, is made amplifiable by a single primer for SNP analysis in an array platform.

Holly Hilton from Roche talked about high- and low-throughput quantitative RT-PCR applications in pharmaceutical research. Their single target expression profiling (STEP) process generates expression profiles of hundreds of tissues and cell lines from Roche's biobank of > 3000 tissues. The STEP is streamlined to, for example, validate targets. For smaller projects, a more flexible and customizable Taqman Express Service (TES) process has been developed to validate key experiments and tests of new cell lines.

Lilian Yengi from Wyeth Pharmaceuticals presented applications of PCR-based technologies in drug safety and metabolism: integration of microarray, RT-PCR and enzyme activity approaches. They have designed a custom drug metabolism Affymetrix microarray, and also real-time PCR assays to quantify P450 mRNA levels in any given sample. One aim was to test if cytochrome P450 (CYP) mRNA levels in the skin can be a surrogate for liver CYP activity. Another aim was to study the induction of drug metabolism enzymes. A number of case studies were presented and one of the general findings was a direct correlation between mRNA, protein, and activity data.

Next, Michael Myers from the Center of Veterinary Medicine at the FDA talked about the development of PCR-based assays for the detection of prohibited and exempt animal-derived materials in animal feed. The reason for the FDAs concerns are epidemiological evidence that associate bovine spongiform encephalopathy (BSE) with animal feed, and epidemiological data associating with the variant form of Creutzfeldt-Jakob disease (vCJD) in man. The 1997 feed ban requires specifying the species and also tissues of all imported meats. December 2000 the US Department of Agriculture (USDA) banned the importation of all rendered or processed animal material of European origin. The main methods used for testing are feed microscopy, immunochemical assays, and PCR. By PCR, a sample can be analyzed within 24 h, of which 16 h are needed to solubilize the feed material. The rates of false-positive and false-negative samples characterized by the PCR approach were ~ 1%.

The last speaker on this day was Charlie Barnett from Healthspex, TN, USA, who described the development of a new, inexpensive system for the simultaneous detection of multiple genetic mutations in a range of clinical and non-clinical settings. The system is a 5 × 5 array of 1 mm photodiodes. A suitable slide containing dyed samples is illuminated by a laser and the fluorescence is read out. The system can be made very small and is, therefore, suitable for field use. The overall protocol entails the following steps: DNA isolation; PCR; preparation of slide; hybridization of slide; washing; and detection in Healthspex detector.

In celebration of 20 years of PCR

The first of the keynote presentations on the Wednesday was by Norman Anderson from the Viral Defense Foundation. He described the global defense consortium, which has the objectives of routinely detecting and identifing both new and known infectious agents in circulation in large human populations, and to make possible rapid and effective responses. Viruses, even in trace amounts, can be isolated from tissues in a large scale by zonal centrifugation due to their distinct sedimentation coefficients, which discriminate them from other cellular components. Combining centrifugation with microbanding viruses in 100 l pooled samples can be concentrated to few microliters. These pooled samples could be taken from the 500 l of blood serum discarded every week by large diagnostic laboratories in the US. By cloning and shotgun sequencing, a large number of viral genomes could be sequenced simultaneously. Such an approach would make it feasible to monitor changes in the human viral flora on a regular basis.

Fubao Wang, from Vaccine & Biologics Research, Merck Research Laboratories, PA, USA, talked about the application of PCR technology in vaccine product development. He focused on the development of a replication defective adenovirus 5 HIV I gag (Ad5HIVgag) vaccine. The adenovirus is a non-enveloped 36-kb linear DNA virus that replicates in the nucleus of host cells. Deletion of the transforming early region 1 (E1) segments disables viral replication and diminishes viral gene expression. Such an adenovirus is only produced in PER.C6 cells that provide E1 in trans. An optimized HIV gag is inserted in the vaccine. There are > 47 human adenovirus serotypes and most adenovirus infections are subclinical. A PCR system has been developed to distinguish the Ad5HIVgag vaccine from the human serotypes in order to monitor vaccine levels during all stages of vaccine development, including research, preclinical development, clinical trials, and commercial production.

Peter Dailey, the Director of the Infectious Diseases Department at Roche Molecular Systems, then provided a historical survey of PCR diagnostics: the continuing revolution. It was a very entertaining talk starting with the very beginning of Kary Mullis inventing PCR, going through the development of both instrumentation and real-time PCR.

Indira Hewlett, the Chief of Molecular Virology Laboratory, at CBER/FDA,

talked about PCR technology for HIV testing – a regulatory perspective. The first report of HIV-1 DNA by PCR was published in 1987, and since 1988, PCR has been used to test for HIV in blood samples and plasma. In 1996, the FDA recommended approval for the first PCR assay for HIV viral load. It became incorporated into the standard of care and the guidelines for management of HIV patients. In 2002, the first PCR tests were approved to identify drug resistance. PCR methods are important to detect HIV in antibody-negative blood donations, which may have been collected during the window period, which is the time between exposure to an agent and detection of the infection with screening tests. Using antibody screening assay, the risk of missing HIV was 1 in 420,000–600,000. Introducing HIV p24 antigen screening in 1996 reduced the risk to 1 in 1.3 million. The WHO international working group for the standardization of gene amplification techniques (SoGAT) released the final guidance for tests to detect HIV-1 and HIV-2 in 1999. This has brought down the risk of HIV in blood screens to 1 in 3 million. Using microarray technology targeting the ENV gene variable region, different isolates can be monitored in a cost-efficient manner.

A selected oral poster presentation on the multiplex detection of plant pathogens by padlock-based universal multiplex detection arrays (pUMA) was delivered by Cornelis Schoen from Plant Research International, the Netherlands. pUMA is based on a padlock probe (PLP), which in its linear form has the first part of the target sequence, the universal forward primer, the universal reverse primer, the ZIP code, and the second part of target sequence. After hybridization to the target, the PLP is closed by ligation, and, after the removal of linear PLP by washing, the immobilized PLPs are PCR amplified. The PCR products can then be separated by hybridization to an array of adaptors with complementary ZIP codes. The system has been shown to detect six different targets over an 100-fold concentration range, and a multiplex system for 28 pathogens is being developed.

The final speaker of the morning session was Nancy Leysens from the Pioneer Hi-Bred International. She talked about the applications of high-throughput quantitative PCR for the genetic characterization of plant tissue in the Biotech commercialization pipeline. In the breeding process Pioneer uses Q-PCR to screen events for copy number, expression, and integrity of the insertion event, to screen events for zygocity, and to screen seed pools for purity. To improve throughput, most assays has been converted to Taqman end point assays. Comparing a marker gene with an endogenous control in duplex assays they always distinguish one from two copies, and with good accuracy from three copies.

Small chips for small genomes

In the section devoted to biochips. Robin Liu from Applied NanoBioscience Center at the Arizona State University, AZ, USA, presented a fully integrated microfluidic and microarray biochip for DNA analysis. The chip components included paraffin-based microvalves that use a plug of wax as an actuator; pumps based on an air pocket attached to a heater that expands the air to pump small volumes of liquid, and another pump based on pair of electrodes for the electrolysis of water, which generates hydrogen and oxygen gases in order to move larger volumes of liquid. Liquids are mixed using sound waves to vibrate air bubbles trapped in the mixing chamber. Starting from a 1-ml blood sample, the integrated chip performed concentration purification, lysis, PCR and detection within 3 h.

Jizhong Zhou from the Environmental Sciences Division, Oak Ridge National Lab, TN, USA, was the next speaker. He described their work on developing microchips for bacterial genomes to define gene functions and regulatory networks on the genome level. Gene expression patterns are monitored under different growth conditions to identify genes involved in processes, such as metal reduction in Shewanella putrefacines MR-1. They are also isolating and characterizing extremophilic metal-reducing bacteria, such as hyperthermophilic bacteria, that can produce magnetite at the temperature range of 50–115°C, and psychrophilic metal-reducing bacteria from a variety of cold environments.

Hong Ge from the Infectious Diseases Directorate, Naval Medical Research Center, MD, USA, talked about the genomic differences between the virulent strains versus avirulent strains of *Rickettsia prowazekii*. *R. prowazekii* is the etiologic agent of typhus, which infected > 20 million people and caused the death of > 3 million people during World War I (WW1). Several millions died of typhus during WW2 and, as late as 1997, > 100,000 were infected in Burundi. Several *Rickettsia* strains are considered biothreats. The pathogenic mechanism is unknown and the objective is to explore genetic difference between virulent and avirulent strains. Functional categories were identified for 25 out of 885 genes by microarray hybridization. Using real-time PCR, the expression ratios between the virulent and avirulent strains were determined for the 25 genes.

Michael Egholm from 454 Life Sciences then presented whole genome sequencing by the synthesis of bacterial genomes on credit-card-sized PicoTiterPlates (PTP). In the 454 approach, the genome is first fragmented and adapters are attached to each fragment. The fragments are then captured on beads with complementary primers and are PCR amplified in an emulsion with oil, such that the PCR product is contained and one of the strands is trapped on the bead. The beads are then deposited on the PTP at a low concentration so that each well contains no more than a single bead. Three plates are currently available with 0.3–1.6 million wells. Excess of sulfurylase/luciferase beads is then added together with pyrosequencing enzymes, and the plate is placed in the camera. The trapped DNA strands are sequenced by synthesis and formation of pyrophosphate leads to chemiluminescence. Nucleotides are sequentially flowed over

the PTP and the camera registers when and in which wells the nucleotides generate light. Finally, the genome sequence is assembled from the different sequence readouts by bioinformatics. Recently, 454 sequenced a bacterial genome of 2.8 Mb using this technology.

The last speaker of the meeting was Nick Cirino, the Director of the Biodefense Laboratory at the Wadsworth Center, NYS Department of Health, USA. Cirino talked about multiplex select agent diagnostics for public health laboratories. Current primary biothreat agents are anthrax, *Clostridium botulinum* toxin, tularemia, smallpox, Ebola, plague, and ricin. The diagnostic platforms are culture strategies, imaging, biochemical methods, immuno-based methods, and nucleic acid-based methods. Major efforts in their laboratory is being spent on developing multiplex (quadruplex) Q-PCR assays for multitarget analysis of single organism for higher confidence in interpretation, and for multi-organism analysis in precious samples. Using combinatorial assay design, nine organisms can currently be tested for in six quadruplex assay reactions, which include non-template control, inhibition control, positive control, and two targets per organism.

Outlook and summary

The meeting was excellent, covering all steps of gene expression analysis, as well as considerations on high-throughput and examples of important applications. Clearly, what is most important for successful analysis is the quality of the sample material. When studying fixed samples extracting good-quality RNA becomes an issue. DSP fixation seems to preserve RNA better than formalin and ethanol, and random priming works better than poly(dT) when transcribing the RNA of poor quality. Any freshly prepared RNA is rapidly degraded if RNases are not inactivated. This can be done by salting out or adding storing solution, such as RNeasy from Qiagen [6]. For matrices of low complexity, the cell-to-signal system from Ambion [7] lyses cells and is compatible with both RT and PCR. Still another approach is to use filters, such as those developed by Whatman [8], to purify and store nucleic acids.

Frequently, the collected material must be amplified for analysis. For genome analysis, WGA, as developed by molecular staging [8], is an option. Other approaches are based on fragmenting the genome and adding adapters to it for PCR amplification and subsequent microarray analysis for either SNPs, as developed by Perlegen [9] and Parallel [10], or massive parallel sequencing, as developed by 454 Life Sciences [11]. If the DNA is damaged, an option may be to use the DNA polymerase repair enzyme blend called Restorase from Sigma-Aldrich [12]. For direct parallel analysis of RNA, Genentech's [13] NACA can be used. Alternatively, RNA can be amplified using T7 amplification, or a more advanced variant, such as Ovation from NuGEN [14], could be employed. These methods introduce some bias in the expression pattern, but are good enough for most purposes. The RNA can be analyzed en masse by microarray hybridization, or reverse transcribed to cDNA. The RT yield, however, varies up to 200-fold on the choice of RT, priming strategy, and mRNA target [15]. As long as the same protocol is used and relative gene expression is compared results are reliable, but comparison of data from two labs that use different protocols may be tricky. The dominant technique to quantify cDNA is real-time PCR [16]; though, if heating must be avoided, helicase-dependent amplification from New England Biolabs [17] may be an option.

Many reporter technologies are available for real-time PCR. SYBR Green [18] and the BEBO [14] dyes are available as non-specific reporters. Since the design of the Taqman probe [19], a number of other sequence-specific reporter systems have been developed, many of which do not interfere with the PCR reactions, resulting in higher efficiencies. These include AllGlo from Allelogic Biosciences [20], QZyme from Becton Dickinson [21], LNA primers from Exiqon [22], LightUp probes from LightUp Technologies [23], Hyb probes from Roche [24], Molecular Beacons as developed by Tyagi and Kramer [25], Scorpion® primers from DxS [26], LUX™ primers from Invitrogen [27], and the Primer-Probes from WaferGen [3]. The development of quenchers from companies, such as Biosearch Technologies [28], has widened the spectral window for multiplexing using these probes.

The main problem in real-time PCR is the formation of primer–dimer products, which limits the sensitivity of the assays. Primer–dimer products are formed mainly during the preparation of an assay and can be suppressed using Taq polymerase that is inactive until the PCR reaction is initiated. These hot-start systems can be based on chemical modifications of the Taq polymerase, antibody blends (such as presented here by Becton Dickinson [20]), and the new approach based on reversible competition with a synthetic polymer developed by Eppendorf [29]. Hot-start techniques are particularly powerful in combination with probe techniques, where the probing function is an integral part of the primers, or where the probe has an unnatural backbone and can neither prime nor be a substrate for priming. These systems contain fewer oligonucleotides and form less primer–dimer products. Furthermore, better buffer systems, such as Elixir of Brandeis University, suppress primer–dimer formation. These developments are important for multiplexing, where primer–dimer formation is harder to suppress because of the larger number of primers and also total amount of primers that must be used. Becton Dickinson [20], Bio-Rad [30] and the Wadsworth Center reported excellent quadruplex real-time PCR assay data at this meeting.

High-throughput quantitative gene expression analysis is becoming increasingly important in all stages of drug development, vaccine development, plant breeding, and in biodefense research. Advances in sample enrichment, sample preparation, and preamplification are important steps toward higher throughput. In the case of real-time PCR, further development of multiplex methods will be important, as well as platforms that handle more samples and use smaller sample volumes, such as

those being developed by WaferGen [3] and Biotrove [31]. The large amounts of data generated will require automatic quality assurance systems, such as kinetic outlier detection (KOD) developed at TATAA Biocenter [14]. Software, such as GenEx from MultiD Analyses [2], to classify samples based on the expression of multiple targets will also be needed. Finally, integration of the different steps going from sample to analysis will be important, as well as controlling the work and information flow.

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