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Feature Article

Multiplexing Methods Head to Clinic

Automation Increases the Reliability and Speed of Target Validation

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High-throughput discovery technologies deliver an astounding quantity of data but often the greater challenge is to identify real targets from the vast body of potential hits. Typically, this task requires multiple variations of experimental conditions in hopes of finding meaningful correlations. A new generation of inexpensive and rapid multiplexing technologies aim to ease the burden of target validation and build the bridge into clinical diagnostics.

Analysis of multiple SNPs per gene or multiple epitopes per protein increases the fidelity of the assay and leads to more accurate disease identification, which may directly affect treatment. Automation is the key aspect of multiplex analysis because it minimizes operator errors and investigator's influence on the experiments. Automated multiplexing technologies may soon become commonplace in both diagnostic laboratories and clinical investigations.

A number of speakers looked at the role of multiplexing assays during the "Tenth International Conference on Biodetection Technologies: Technological Responses to Biological Threats," which took place in Atlanta on June 14 and 15, and Cambridge Healthtech's recent "Molecular Medicine Tri Conference" in San Francisco.

"The new generation of multiplexing technologies have to provide a large dynamic range in order to have sensitivity in the sub petagram per mL range, to adhere to a simple and fast protocol with less than two washes, and, finally, to require only a few microliters of sample," commented Pankaj Oberoi, Ph.D., director of scientific services at **MesoScale Discovery** (MSD; www.mesoscale.com). "If we can conserve the sample, eliminate the need for dilutional series, and cover a 3.5 to 4.5 log dynamic range, which is commonly observed with cytokines, we could really open the door for clinical diagnostic applications based on analysis of complex mixtures of molecules."

MSD provides fully developed assay kits for intracellular signaling, secreted biomarkers, and safety toxicology. The company says it utilizes a unique reporter system based on an electrically driven chemical reaction that gives off infrared light (electrochemiluminescence). Detection molecules are labeled with ruthenium, a nonradioactive compound that does not emit light under normal conditions. When electric current is applied, ruthenium undergoes a redox chemistry. Tripropylamine transfers an electron to ruthenium, which in turn emits light at 620 nm.

"At this wavelength, there is little or no quenching or interference from the typical biological fluorescence of cells and cell fragments," continued Dr. Oberoi. "Because the ruthenium conversion reaction requires electrical current, this conversion occurs only within one to 10 micrometers from the bottom of the plate, which is the layer occupied by the capture molecules with the bound analytes. It is so specific, you may not need any washes."

To create these assays, MSD incorporates a miniature circuit board into the plate, where the pair of carbon electrodes serves as a bottom of each well. Capture molecules are spotted directly on the surface of the working electrodes. The carbon surface is able to hold 10- to 100-fold more molecules than a typical polypropylene plastic surface, and it is also suitable for seeding cells, says Dr. Oberoi.

"Carbon is a reactive surface and will work with a variety of capture molecules, including peptides, carbohydrate, and viruses. Another advantage is the ability to use complex matrixes such as sputum or tissue homogenates with only minimal sample prep, whereas systems based on beads and fluidics would be simply clogged," said Dr. Oberoi. The system can detect about 100,000 molecules per spot, and it takes about one minute to read a multiplexed 96- or 384-well plate, reports the company.

Automated Gene-expression Profiling

Xceed Molecular (www.xceedmolecular.com) says it increases the productivity of microarray experiments by processing up to eight arrays in about four hours.

“We saw an emerging need for an automated platform for gene-expression profiling,” said Susan Bromley, Ph.D. vp, diagnostic product development. “The microarray space is transitioning from discovery applications using whole-genome arrays to validation of focused sets of candidate genes. Such experiments require analysis of a subset of genes using multiple experimental conditions or large sample sets.”

Ziplex™ Automated Workstation performs all steps of microarray processing, including data collection and analysis, in an automated format. Each workstation is able to simultaneously process eight proprietary chips.

The 6.5-mm square Flow-Thru Chips® are made out of porous silicon. This unique substrate contains more than 700,000 uniform microchannels per square centimeter. Each microchannel has a diameter of 10 mm and a length of 450 mm. When oligonucleotide probes are printed on the chips, each resulting spot covers and fills about 70 of the individual microchannels, with a total surface area for probe binding a hundred times greater than that achieved on an impermeable, 2-D surface, reports the company. The porous structure allows reagents to cycle through the chip during incubations, reducing hybridization times.

“Many laboratories currently outsource microarray processing and analysis,” says Dr. Bromley. “Our system will allow researchers and, in the future, reference labs and hospitals to bring microarray technology in-house.” The Ziplex™ Workstation will be available for research use later this year. Currently, the company is trying to obtain FDA clearance for its instrument and diagnostic kits.

“Many infectious diseases present similar symptoms. Doctors commonly prescribe a broad spectrum antibiotic without an exact diagnosis. A rapid test is available only for Streptococcus, and typical lab analysis for other infections may take up to five days,” said Charles Daitch, Ph.D., president of **Akonni Biosystems** (www.akonni.com). “Ideally, a doctor would screen a patient’s sample for multiple infectious diseases using a rapid, simple-to-use, low-cost test.”

Akonni developed TruArray™, arrays of 3-D microdrops consisting of a proprietary hydrogel. The diameter of each drop is 100 microns. DNA or antibodies are anchored to the gel, creating a micro test tube. The arrays are embedded into microfluidic devices.

“We have developed a simple and straightforward microarray production method,” added Michael Farmer, chairman of Akonni’s Board. “All components of the micro test tube are mixed together, spotted on solid support, and UV-polymerized. As a result, we could decrease the cost to less than \$10 per array for identifying multiple pathogens. It takes us only 30 days to produce and validate a test for a new microorganism.”

The company plans to initially market the arrays via public health disease surveillance labs. The Akonni system may be useful for early identification of pathogens where symptomology by itself is insufficient. The system, due to be released at the end of 2008, will include a portable reader device and credit card-sized disposable microfluidic card with embedded TruArray. The card, loaded with a small sample of blood, urine, or saliva, is placed in the reader, which automatically processes the sample.

“We are also developing on-chip PCR to increase sensitivity of detection to about 100 colony-forming units per milliliter,” added Dr. Daitch. “Each microdrop would contain a covalently tethered pair of specific primers. Since primers are spatially isolated, we would eliminate primer interference and achieve specificity of detection of five standard deviations above background. We can achieve highly multiplexed amplification impossible in solution-phase PCR.”

In a recently completed two-site trial, Akonni compared TruArray technology for identification of drug-resistant tuberculosis with a standard eight-week culturing procedure. Eighty-eight SNPs from six genes associated with multidrug resistant tuberculosis were anchored in the gel drops. DNA was obtained from sputum samples, amplified, and hybridized to the arrays. The TruArray procedure demonstrated similar sensitivity and specificity as culturing, but completed the process within several hours.

Molecular Diagnostics Workstation

The Verigene System from **Nanosphere** (www.nanosphere-inc.com) is a fully automated molecular diagnostics workstation able to process up to four samples at the same time, according to Nanosphere. “Our goal is to simplify molecular diagnostics testing and at the same time to deliver a high degree of accuracy,” said William Cork, CTO and vp of research and development. “In clinical trials our system demonstrated 100% specificity of SNP identification with minimal operator involvement.”

At the heart of Nanosphere technology is the 13–15 nm gold nanoparticles. “We chose gold because of its stability in the colloid form and easy functionalization with biomolecules,” continued Cork. “For example, DNA-derivatized nanoparticles are stable at room temperature for several years.”

In a typical assay, capture oligos are immobilized on a glass slide and hybridized with a target DNA. Gold nanoparticles coated with complementary oligos serve as detection probes. “Each nanoparticle contains several hundred oligos. Therefore, if one interaction with the analyte is destabilized, another oligo will instantly re-hybridize. This results in a sharp melting transition, compressed to just one degree Celsius,” continued Cork.

To amplify the signal, each gold particle is grown to 200 nm in diameter by coating it with silver. A 200-nanometer silver-enhanced nanoparticle is approximately 1,000-fold brighter than a fluorophore. Light or photon scatter is used to detect the Au-Ag probes. “The lower limit of SNP detection by Nanosphere’s method is 40 nanograms per microliter. We amplify the signal rather than the target. Thus, we eliminate fidelity and contamination issues,” added Cork.

Mid-range Analyte Arrays

Bio-Rad (www.bio-rad.com) utilizes a bead-based xMAP™ technology originally developed by **Luminex** (www.luminexcorp.com). Each 5.6-µm polystyrene microsphere is internally labeled with a specific combination of a red and far-red fluorescent classification dye. Ten different concentrations of each color create 100 classes of beads, each with a unique spectral address. Each class of beads can be conjugated with its own capture molecule. A desired number of conjugated bead classes are pooled in a well of a 96-well plate and incubated with a sample. A fluorescent reporter, phycoerythrin (PE), is used to detect bound analytes.

The detection of captured protein, peptide, DNA, or RNA is performed in the Bio-Plex Suspension Array System, a modified flow cytometer. The beads pass single file through a narrow flow cell, where two lasers excite the beads. The red laser (635 nm) excites the classification dye in each bead, identifying its spectral address. Bio-Plex Manager software correlates the address with the capture molecule assigned to the bead. The green laser (532 nm) excites the PE reporter molecule, which allows quantitation of the captured targets of interest.

The system is programmed to count beads until it reaches a predetermined number, usually 25, 50, or 100 per class, at which point the system calculates a median fluorescent intensity of each bead class. Standard curves can provide relative quantitation of concentration similar to ELISA. “In its current format, xMAP technology is able to quantify up to a hundred nucleic acid species in each sample and 96 samples in parallel. The effective upper limit on immunoassays is typically about 30 to 35 plex, due to inevitable cross-reactivity considerations. This format satisfies multiple applications such as clinical diagnostics and research, environmental analysis, animal health, and basic life science research,” said Dennis Bittner, Ph.D., new technologies manager.

“There is a big analytical gap between single-analyte ELISA and thousand-analyte microarrays. The critical need is in measuring analytes in the range of dozens, and xMAP technology fits the bill well.” The sensitivity of detection is in the 1–10 pg/mL range for proteins and hundreds of copies for nucleic acid applications, with a dynamic range that is close to four orders of magnitude, according to the company.

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