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Target Enrichment for Clinical and Molecular Diagnostics

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Target-enrichment technologies offer increasingly powerful and cost-effective ways to detect various pathogens, disease biomarkers, and toxins by molecular amplification or direct capture in clinical diagnostics, basic research, and industrial applications.

The latest developments in the field were discussed earlier this year at two conferences—CHI's "Sample Prep and Target Enrichment in Molecular Diagnostics" and the Knowledge Foundation's "Sample Prep".

Mark Eshoo, Ph.D., director of new technology at Ibis Biosciences, a subsidiary of Abbott Molecular, talked about an isothermal amplification, broad-range PCR, electrospray ionization mass spectrometry (IA/PCR/ESI-MS) assay that allows early diagnosis of Lyme disease. The assay works by detecting the tick-borne pathogen Borrelia burgdorferi directly from whole blood, cerebrospinal fluid, or other clinical specimens. To achieve the necessary sensitivity, the assay selectively enriches the target Borrelia sequences in large sample volumes.

"The Centers for Disease Control and Prevention reports that there are an estimated 300,000 new Lyme disease cases a year in the United States. If left untreated, this pathogen can lead to chronic long-term infection," said Dr. Eshoo.

"The optimal time to treat Lyme disease is at the onset of symptoms," said Dr. Eshoo. "Currently, serological tests have low sensitivity early in the infection due to the biologically delayed immune response, and as a result, the tests cannot distinguish active infections from prior exposures."

"Abbott has been working to develop a method for the direct detection of the bacteria that causes Lyme disease, Borrelia burgdorferi. To detect the bacteria, Abbott has developed methods for using large volumes of blood and an isothermal amplification technique that we used to increase our assay sensitivity," added Dr. Eshoo.

The isothermal amplification, an effective and simple non-PCR DNA enrichment technique, was added to the existing PCR/ESI-MS assay. The study targeted eight Borrelia loci by using 50 primers per target (400 primers in total) in conjunction with a strand-displacing DNA polymerase to increase the target enrichment efficiency in the sample DNA extracted from 1.25 mL of whole blood.

The IA/PCR/ESI-MS assay takes eight hours and can detect Borrelia burgdorferi prior to an active infection. These capabilities allow doctors to diagnose erythema migrans and acute Lyme disease early in disease progression before seroconversion.

Next-Generation Sequencing

Jude Dunne, Ph.D., vp of product development at WaferGen, described the SmartChip TE system, which uses next-generation sequencing (NGS) to provide a target-enrichment solution for clinical and translational research.

"The system uses a singleplex PCR enrichment strategy, with a single reaction occurring in each of several thousand wells on a SmartChip TE panel in less than three hours," said Dr. Dunne. "At the end of the enrichment, all the amplicons are extracted into one pool ready for sequencing. In situ addition of the required sample-identifying barcodes and sequencer-specific adapters … further simplifies the workflow and reduces cost."

Singleplex PCR can allow the user to optimize individual assays on the chip, with the possibility of monitoring each reaction in real time. According to Dr. Dunne, WaferGen's custom assay designs have a higher probability of working on the first iteration, unlike some multiplex technologies for which several rounds of optimization have become a common practice.

The SmartChip TE system consists of custom SmartChip TE panels, a desktop sin-gle-sample dispenser, and a PCR cycler. The system's level of sensitivity with routine coverage is >99% of the targeted regions, and its uniformity of coverage is >98% at >10% of the mean.

"We are adding to our primer design pipeline by including designs for smaller am-plicons suitable for FFPE samples, which are critical for cancer-related clinical tests," said Dr. Dunne. "In addition, a higher throughput SmartChip TE system with a multisample dispenser will be offered in the near future to enable enrichment of multiple samples on one chip.

Those will be two main product improvements that will allow us to address the needs of the vast majority of clinical and CLIA-certified labs."

“We feel that our technology gives our customers an edge in detecting all the genetic variants, obtaining the best uniformity and most complete coverage compared to other technologies, which is of critical importance for clinical tests that will be used by doctors to make patient-care decisions,” said Dr. Dunne.

Microbiome Analysis

According to Fiona Stewart, Ph.D., product development manager at New England Biolabs, customers asked the company to develop a better method for separating target microbial DNA samples from host DNA, the main contaminant. The method needed to be good enough to enable an accurate microbiome analysis.

“What we came up with was an idea to separate those two types of DNA based on methylation pattern. Eukaryotic DNA is generally methylated, and microbial DNA is generally not,” said Dr. Stewart. The separation is achieved by capturing the methylated host DNA on methyl-CpG-binding domain protein 2 (MBD2), immobilized on magnetic protein beads.

Erbay Yigit, Ph.D., applications and product development scientist, used the kit to analyze the microbiome of human saliva. “Once we pull down the methylated host DNA, what remains is the supernatant,” said Dr. Yigit. “Most of the host DNA goes in the beads fraction. We are primarily interested in sequencing prokaryotic DNA.” The prokaryotic DNA sequences are verified against the Human Oral Microbiome Database (HOMD).

The host DNA fraction can be released from the beads and analyzed separately. “We are not only enriching the supernatant, which contains the prokaryotic DNA, but we are also enriching the host DNA,” said Dr. Yigit.

The company’s NEBNext Microbiome DNA Enrichment kit has been used to analyze human, fish, plant, and arthropod microbiomes. It also selectively enriches the organellar DNA from mitochondria and plant chloroplasts, which enables a more efficient genome sequencing and mutational analysis. Dr. Stewart said that the company plans to develop the viral enrichment technology in response to demand in the research community.

“The variety of applications seems pretty broad, but what all of these people have in common is the same problem—they want to be able to sequence the microbial DNA in their sample, and previously they had not been able to do that,” said Dr. Stewart. “Being able to separate microbial DNA is bringing real changes.”

Proteomics-Based Immunoaffinity Enrichment

Modern discovery experiments in proteomics can identify hundreds of thousands of candidate biomarkers. However, this wealth of information is known to pose its own challenges. As indicated by Jeffrey Whiteaker, Ph.D., director of proteomics in the Paulovich laboratory at the Fred Hutchinson Cancer Research Center, “There is a very little technology available for following up on those quantitative studies.”

To address this issue, the Paulovich laboratory has developed selected/multiple reaction monitoring (SRM/MRM) for targeted biomarker discovery. “We have coupled the immunoaffinity enrichment of peptides with mass spectrometry for their quantitation,” said Dr. Whiteaker.

The sample contains the targeted peptide and the stable isotope internal standard, both of which are enriched with the same antibody before going into the mass spectrometer. The SRM assay employs the combination of selecting a precursor master charge and a fragment master charge, which is called the transition, to filter out the targeted fragment for final analysis.

The lab developed new assays targeting breast cancer and compared them to the existing assays. The plasma samples were arranged in different concentrations and multiplex levels reaching the maximum of 50. The increase in multiplexing did not affect the sensitivity of the assay or the running time per sample.

“If you look at analyzing the sample on per-analyte basis, multiplexing offers tre-mendous savings. In building the assays, requiring only one antibody inherits some cost savings over multiple antibodies,” said Dr. Whiteaker. In addition to traditional polyclonal antibodies, the Paulovich laboratory is planning to explore other affinity reagents, including recombinant antibodies or aptamers.

Developing biomarker and clinical assays is not a sole focus of the lab. “ We are also looking at developing new technologies for more fundamental studies in cell lysates, which can be perturbed by various treatments or conditions. Biologist can assemble a multiplex targeted assay and interrogate many samples by using that,” said Dr. Whiteaker.

Nanofiber-Based Sample Preparation

An alternative approach to the DNA and protein-based target enrichment techniques is the nanofiber-based sample preparation from the Bioanalytical Microsystems and Biosensors (BMB) Laboratory at Cornell University. The lab has fabricated poly(vinyl alcohol) (PVA) nanofiber mats by integrating functionalized electrospun nanofibers into microfluidic chambers. They are intended to serve as on-chip bioseparators in complex µTAS and point-of-care diagnostic devices.

“Once we mastered the pure fabrication part, we then looked into how the different surfaces could be exploited for different types of sample preparation. The very first surfaces that we used were negatively and positively charged nanofibers,” said Antje Baeumner, Ph.D., principal investigator at the BMB laboratory.

The PVA nanofiber mats were developed in collaboration with the laboratory of Margaret Frey, Ph.D., an associate professor of fiber science and apparel design at Cornell University.

As one would expect, in theory, the positively charged nanofiber mats work equally well with negatively charged liposomes and Escherichia coli cells. The analytes can be released from the nanofibers by changing the pH of the buffer solutions, making the mats reusable. The binding is detected by fluorescence imaging, but Dr. Baeumner wants to develop more specific verification methods.

The lab has started working on a paper-based technology to expand their nanofiber-based approach to a variety of diagnostic platforms. “We have developed de novo nanofiber mats that work at least as well as nitrocellulose, but had many more capabilities than typical electric flow assay membrane,” said Dr. Baeumner.

Currently, the lab is developing the nanofiber mats with a larger surface area and an embedded recognition element, so they can discriminate between different types of pathogens, endotoxins, and analytes.

“What we would like to accomplish is the ability to use these nanofibers in a way that would let us avoid molecular amplification as much as possible,” said Dr. Baeumner.