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Feature Article

Capitalizing on microRNA Expression

New Tools Improve Sensitivity, Throughput, and Sample Size

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The enormous potential of miRNAs is driving new technologies for research, diagnostic, and therapeutic applications. miRNAs, a class of small noncoding RNAs of 19–30 nucleotides in length, regulate approximately 30% of all human genes. Elaborating miRNA-expression patterns may hold the key to increased understanding of cell development, metabolism, and a range of diseases.

A miRNA- and siRNA-focused Keystone Symposium held earlier this year highlighted some of the progress in the field. Improvements reviewed included more sensitive and specific miRNA profiling using synthetic RNA/DNA probes or optimized native probes. Other advances featured aimed at enhancing throughput using a bead-based multiplex assay and at creating miRNA profiles from single cells or limited biopsy material.

miRNA Profiling

“At the Keystone Symposium, there was a great deal of optimism, especially about the potential of miRNA-related biomarkers associated with cancer and developmental biology that are just beginning to be discovered and characterized. Everyone is eager to understand what can be revealed by different cells’ unique miRNA-expression patterns,” said Hui Wang, Ph.D., senior research scientist at **Agilent Laboratories** (www.agilent.com).

Patterns of miRNA expression provide a snapshot of cellular activity and can contribute to the identification of disease biomarkers. Additionally, profiling miRNAs may help decipher their role in cancer. Many researchers use microarray technology to monitor and evaluate miRNA expression in normal and diseased tissue. As a result, Agilent has developed a microarray tool to characterize miRNA-expression patterns and recently introduced a commercially available human miRNA microarray.

“The Agilent Human miRNA Microarray platform is quite distinct from others,” said Dr. Wang. “Our platform uses low sample input, obtaining miRNA-expression profiles directly from 100 nanograms of unfractionated, unamplified total RNA. This is possible because we have developed an efficient, direct labeling method, coupled with a novel probe design, that is capable of both size and sequence specificity.

“Specificity for mature miRNAs is aided by the secondary structures of our probes. Our sequence specificity is greatly enhanced by our probe-selection strategy, where the melting temperature of each microarray probe target miRNA complex is experimentally determined.

“The Agilent microarray platform, which consists of high-quality DNA probes directly synthesized on a glass slide surface with an ink jet printer, has array-to-array reproducibility that is extremely high. It also allows easy updates of the probe content to accommodate the growing miRNA database. Our current platform enables sensitive and highly reproducible measurements and is capable of a linear dynamic range of five orders of magnitude.”

Locked Nucleic Acid Technology

One of the challenges associated with using microarray technologies for miRNA profiling is the different melting temperatures of miRNAs, which make it difficult to establish optimal hybridization temperatures between miRNAs and capture probes immobilized on the array.

“The approximately 450 capture probes used for miRNA microarray analysis are short and their optimal melting temperatures can vary widely. To obtain accurate and sensitive hybridization of miRNAs on microarrays, we developed a locked nucleic acid (LNA) approach that uses modified oligonucleotides as capture probes,” said Martina Muckenthaler, Ph.D., head of molecular medicine in the department of pediatric oncology, hematology, and immunology at the University of Heidelberg in Germany.

The LNA-modified capture probes are synthetic RNA/DNA analogs that have increased thermostability in nucleic acid duplexes. Further, they can be designed to have uniform melting temperatures by simply adjusting the LNA content and length of the capture probes.

“This is important because it will enhance sensitivity and accuracy of the miRNA expression signature. For example, low-abundance miRNAs are more easily monitored compared to normal DNA-based probes,” Dr. Muckenthaler explained. “Additionally, mismatches in miRNAs are discriminated for. This increases the hybridization specificity for a related miRNA family member, which often varies by as little as a single nucleotide.”

In developing their microarray platform, Dr. Muckenthaler and her team optimized RNA-preparation protocols, coating chemistries for glass surfaces, chemical modifications of capture probes, as well as labeling and hybridization protocols.

“This technology has many applications,” she added. “It is one of the best tools currently on the market for monitoring miRNA expression because it allows detection in any given biological sample. In the case of cancer, our focus is on T-cell leukemias. We currently profile 30 to 50 samples and get 400 miRNAs to monitor. The 20 to 30 miRNAs expressed will have different levels in different patients. Then, the challenge is to correlate expression with clinically relevant parameters. Is it a good or bad indicator for an early response to treatment and can we use this to help refine treatment?”

This LNA technology is commercially available from **Exiqon** (www.exiqon.com).

High Throughput with miRNA Beads

LNA technology is also being applied by **Luminex** (www.luminexcorp.com) in a new high-throughput, bead-based multiplex assay for miRNA-expression profiling. The company combined LNA with its xMAP technology to create the FlexmiR profiling platform.

“We just completed development of this flexible and open platform that essentially hits a sweet spot with both the number of miRNAs assessed and the number of samples that can be analyzed,” reported Keld Sorensen, Ph.D., director of R&D.

Luminex created the bead-based profiling method as an attractive alternative to the planar arrays. “The FlexmiR uses a fast and straightforward procedure in which researchers biotinylate the 3’ ends of total RNA, hybridize the labeled RNA to the LNA capture probes that are coupled to fluorescently dyed xMAP beads, tag the captured biotinylated miRNAs with streptavidin phycoerythrin, and, finally, assess the samples on our analyzer,” explained Dr. Sorensen.

The system excels over current technology that can only measure a limited number of miRNAs or can measure a lot of miRNAs but on a limited number of samples, noted Dr. Sorensen. “With this technology, you can do both. You can measure up to 100 unique assays in each well of a 96-sample microtiter plate, and you can run up to five plates per day, so this is true high-throughput multiplexing.

“A number of people believe that while there are lots of miRNAs, it is likely that there are only a small number (~20) that are actually important in disease states. Once you establish a set of miRNAs for the disease condition, then you can quickly test large numbers of samples,” Dr. Sorensen added.

For the future, he believes that “researchers will be able to mix and match the set of miRNA beads they want. That’s where the field is moving.”

Profiling Single Cells

The ability to create an miRNA profile from a single cell has major ramifications, particularly with neuronal and stem cell research, according to Caifu Chen, Ph.D., director of assays R&D at **Applied Biosystems** (www.appliedbiosystems.com).

“Our company made a major breakthrough in single-cell gene-expression analysis,” he reported. “We developed RNA-profiling technologies that need only miniscule amounts of RNA for very reliable, reproducible, and quantitative PCR.”

The Multiplex RT for TaqMan® MicroRNA Assays allows profiling of both miRNAs and mRNAs from the same cell. “What makes this process unique and possible is a multiplex preamplification step that amplifies cDNA with primer sets before specific target amplification. This gives researchers the needed sensitivity to work with extremely small samples such as single cells. An added benefit is that this does not introduce amplification bias,” Dr. Chen noted.

How does it work? A three-step protocol enables researchers to quantify only mature miRNAs. A multiplex reverse transcription (RT) reaction with a pool of approximately 450 stem-loop RT primers is used to convert RNA to cDNA. Then, the cDNA product is subjected to multiplex preamplification. Finally, individual TaqMan reactions are performed using arrays of TaqMan MicroRNA Assays in 384-well plates that accurately determine the level of miRNA expression.

Another application of the technology is the generation of miRNA profiles from biopsy material for clinical research. “Often one has only a small amount of genetic material from patient biopsies. Being able to create miRNA profiles is significant for these kinds of applications. Importantly, our protocol is also suitable for formalin-fixed, paraffin-embedded samples,” added Dr. Chen.

He feels that real-time PCR is better suited for capturing miRNA-expression information as compared to microarrays. “Because of the increased assay sensitivity, you can start with 10-fold less RNA and still detect miRNAs expressed at a lower level. In other words, TaqMan miRNA assays will help researchers see more miRNAs,” Dr. Chen explained.

Other advantages he highlighted for the technology include a broader dynamic range that benefits the widely varying levels of miRNA, greater specificity for discriminating closely related miRNAs, and reduced sample consumption.

Clinical Applications

Although siRNA therapeutics are now in clinical trials, miRNA therapeutics will soon follow, said Andreas G. Bader, Ph.D., senior scientist, Asuragen (www.asuragen.com). “miRNAs are emerging as significant players in the therapeutics arena.”

Why are miRNAs good drug targets? The answer, as relates to cancer therapeutics, is that miRNAs appear to contribute to malignancies similar to conventional oncogenes and tumor suppressors.

“miRNAs are natural molecules required for the regulation of intracellular processes in normal cells that, when deregulated, can lead to tumorigenesis,” Dr. Bader explained. “The role of miRNAs in cancer development is explained by the observation that miRNAs regulate many oncogenes and tumor suppressors. miRNAs are frequently downregulated or lost in cancer. Therefore, restoring the normal expression of miRNAs by administering synthetic miRNAs might be the most appropriate remedy. Since we replace what has been lost, we anticipate no or minimal cytotoxicity.”

Asuragen has developed therapeutic miRNAs that reduce the tumor burden in preclinical animal studies. “We

are excited about these results and are moving some of our leads toward clinical trials,” added Dr. Bader.

Asuragen also offers a variety of pharmacogenomic services. Among these is the DiscovArray™, a comprehensive miRNA expression-profiling platform. The DiscovArray™ contains probes that are specific for more than 13,000 annotated and predicted human miRNAs, according to Dr. Bader.

“miRNA therapeutics for cancer are just the beginning,” Dr. Bader concluded. “We have also identified miRNAs that appear to contribute to other diseases including Alzheimer’s, diabetes, and HIV infections. We expect to continue to make rapid progress in this young field.”

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