

High-throughput Screening Assays: Managing microRNA Expression Profiling Using a Bead-based Multiplex System

ALTHOUGH ONLY RECENTLY IDENTIFIED AS A NEW CLASS OF MOLECULES, microRNAs HAVE EMERGED AS A ONE OF THE MOST PROMISING AREAS OF RESEARCH TODAY. RECENT DATA FURTHER SUGGEST THAT MEDICAL CONDITIONS, SUCH AS CANCER, CAN BE ASSOCIATED WITH SPECIFIC microRNAs PATTERNS. IDENTIFYING THESE PATTERNS MAY LEAD TO THE DEVELOPMENT OF TARGETED TREATMENTS.

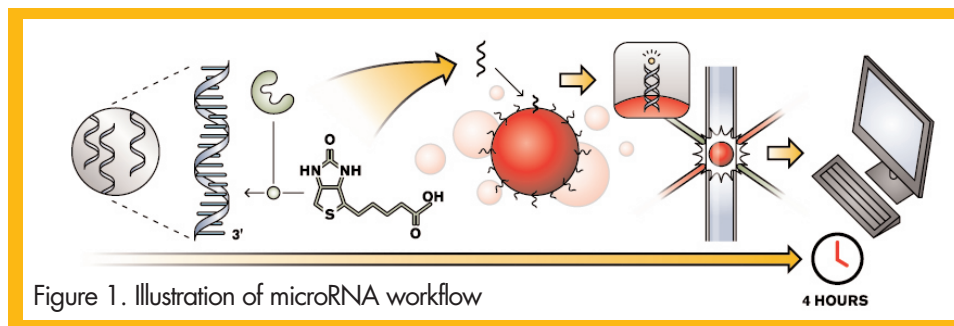
Studies indicate that microRNAs, which are a class of non-coding regulatory RNA molecules that affect gene expression by binding to 3'-untranslated regions of messenger RNAs (mRNAs), may regulate as many as one-third of the genes within the genome and influence a wide range of biological activities and cellular processes, including cellular proliferation, maintenance, and apoptosis; differentiation of cell lines; developmental patterning and timing; and carcinogenesis.

MicroRNA analysis can be challenging for researchers because the database of identified microRNA is expanding quickly. Also, microRNAs are short sequences ranging from 17–23 nucleotides in length that are highly similar to each other in sequence with inherently different melting temperatures.

As researchers study microRNA sequences and focus on critical, relevant microRNA, they require technology specifically designed for microRNA analysis that can be applied to screening against a broad panel of targets and used for focused multiplexing of microRNA targets and patterns of interest against numerous samples consistently and efficiently. This technology also must easily expand or subtract feature sets and process these features against large banks of samples.

Hard-coded planar arrays are at a disadvantage in offering the customization needs of emerging microRNA research, but a combination of a bead-based array and T_m-normalized locked nucleic acids (LNA) offers a solution for the challenges microRNA research poses. Favorable reaction kinetics of a liquid bead array give faster, more reproducible results than solid, planar arrays. This “liquid array” approach also offers excellent manufacturing and assay standardization due to the nature of the microspheres when compared to competing flat arrays, which are limited by solid phase kinetics.

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LNA is a conformationally restricted nucleic acid analogue in which the ribose ring is “locked” with a methylene bridge connecting the 2'-O atom with the 4'-C atom and increases the melting temperature of the nucleic acid duplex by 2–8 °C per LNA monomer when integrated into one strand. The microRNA detection panels for human, mouse, and rat targets give researchers the ability to measure the expression of microRNA sequences from public databases using total RNA samples without the need for RNA size fractionation or amplification. The general flow of such products, illustrated in Figure 1, allows researchers to biotinylate the 3' end of total RNA, followed by a hybridization step where the labeled microRNA hybridizes specifically to LNA capture probes coupled to microspheres. The detection of the biotinylated microRNA is achieved by the reaction with streptavidin-phycoerythrin (SA-PE) and final read of the samples in a standard 96-well plate on an analyzer.

EXPERIMENTAL DESIGN

For the human panel, more than 300 mature microRNA targets are split across five (5) microsphere pools that allows for use of the same microsphere set or region across multiple wells. In addition to the 60–70 microRNA targets per pool or well, nine (9) multi-purpose controls are included in each well for evaluating attributes such as integrity of the assay, integrity of the assay performance, normalization of signal across the wells for one sample, and normalization of signal across a batched run.

There are two protocols associated with microRNA analysis: labeling and detection. Labeling involves adding a biotin molecule to each RNA target via enzyme ligation. Detection consists of hybridizing the labeled RNA with microRNA-specific LNA probes coupled to microspheres and binding a reporter fluorescent molecule to the hybridized microRNA for detection on an analyzer.

From each human tissue, 30 µg (10 µg per replicate) total RNA was combined in a nuclease-free microcentrifuge tube with labeling buffer, biotin conjugate, a biotinylation enzyme, and nuclease free water. Following mixing and centrifugation, the labeling reaction was incubated at 0 °C for one hour. The labeling reaction was stopped by incubation at 65 °C for fifteen (15) minutes, followed by centrifugation. After labeling the total RNA, the labeled total RNA were combined with microspheres coupled to microRNA-specific LNA probes within sixty (60) wells of a 96-well PCR plate. Nuclease-free water was used for the background control replicates. For best efficiency, the samples are set up on the PCR plate batched by microsphere pool.

Direct hybridization of the microRNA targets to the LNA probes was performed in a thermal cycler and consist-

ed of incubation at 95 °C for three (3) minutes to denature any secondary structures in the reactions followed by hybridization incubation at 60 °C for 1 hour.

Following hybridization, the reactions were washed twice with pre-warmed wash solution using a 96-well filter plate and vacuum filtration to remove unbound products. The reporter molecule, SA-PE, was added to the reactions and incubated at room temperature for fifteen (15) minutes on a plate shaker set at 600 rpm to bind with the biotinylated targets hybridized to the microRNA-specific LNA probes that were coupled to the microspheres. The reactions were transferred to a PCR plate and run on an analyzer at about thirty (30) seconds per sample. The reactions also may be run on the analyzer straight from the filter plate as long as the probe height is properly adjusted for the plate. Total time from start of labeling reaction to final read of the samples was about four and one half (4.5) hours.

RESULTS

The experiment generated a total of 4368 data points in just over four hours. More specifically, 364 data points were generated per sample replicate, yielding a total of 1092 data points for each of the three tissues and for the background control. The median fluorescence intensities (MFIs) for the background control were averaged across the three replicates and later used for background correction of the tissue sample results. The MFIs for all tissues were averaged across three replicates and background corrected.

CONCLUSION

As microRNA research becomes more prevalent and additional microRNAs and microRNA patterns are identified, researchers will need technology that allows for both high density and high-throughput screening. Many traditional technologies that allow for high-throughput applications cannot multiplex many tests at once, while many technologies that enable high-density screening cannot maintain the reproducibility required in high-throughput applications. The flexibility of the products — a combination of a bead-based array and T_m-normalized locked nucleic acids — offers researchers the ability to customize a set of microRNAs while maintaining high specificity and simple workflow. These attributes should prove useful to those interested in focusing research to specific microRNA targets or patterns.

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