

TECHNICAL NOTE

microRNA Profiling with FirePlex™ miRSelect: A Technical Overview and Cross-Cytometer Comparison

FirePlex™ miRSelect is a high-throughput, customizable solution for microRNA profiling across a diverse range of sample classes, with assay readout on standard flow cytometers. In this technical note, we give an overview of our microRNA assay and provide a side-by-side comparison of assay performance on common benchtop flow cytometers. On all systems tested, we demonstrate sub-attomole sensitivity, nearly 5 logs of linear dynamic range, and technical replicate coefficients-of-variation less than 5% for most target levels tested. Currently, miRSelect can be used for the multiplexed detection of up to 68 targets per sample.

As microRNA-based research shifts from discovery to validation, robust methods are needed to profile large sample cohorts. To address this need, Firefly BioWorks has developed a high-throughput method for microRNA analysis for use in research and clinical settings. The miRSelect assay is completely customizable – researchers may design panels to detect any combination of microRNAs annotated in miRbase (from any species) in kits of variable size. MiRSelect has been used to profile microRNAs in human, mouse, rat, and *C. elegans* over a broad range of sample classes including serum, plasma, crude cell digests, and formalin-fixed tissues.

Another feature of the miRSelect platform is that it is a multiplexed assay — it is capable of measuring several targets simultaneously in a single sample. Multiplexing is accomplished by using encoded hydrogel particles. Each particle bears a unique “barcode” that identified the microRNA species being detected on that particle. The particles are manufactured by Firefly BioWorks via Optical Liquid Stamping, a versatile method for microparticle fabrication that allows the synthesis of microscopic structures of virtually any shape and chemical functionality.

The miRSelect assay is performed in a 96-well filter plate, allowing the simultaneous analysis of up to 96 samples. All assay steps are carried out at 37°C or room temperature, with shaking. All reagents are non-toxic, yet provide stringent hybridization conditions for

highly-specific microRNA detection. The basic assay workflow involves four main steps with intermittent rinsing:

1. Addition of particles, Hybridization Buffer, and samples to assay wells, followed by a 90 minute hybridization at 37°C;
2X Rinse;
2. Addition of Labeling Buffer, followed by a 45 minute incubation at room temperature;
2X Rinse;
3. Addition of Reporter, followed by a 45 minute incubation at room temperature;
2X Rinse;
4. Addition of Run Buffer, followed by scanning on a standard flow cytometer.

Including hands-on time, the miRSelect assay takes 3.5 – 4.5 hours from samples to data, depending on how many samples are processed in parallel. The assay workflow is outlined in Figure 1.

Unlike other systems that rely on glass or polystyrene substrates, FirePlex particles are composed of bio-inert poly(ethylene glycol) hydrogels. This unique substrate provides (1) solution-like thermodynamics for optimal sensitivity and specificity, (2) a non-fouling surface, and (3) enhanced capacity for 3-dimensional target capture leading to a greater dynamic range. This substrate, coupled with the miRSelect post-hybridization labeling method, makes the platform ideal for detection in crude samples.



Figure 1: FirePlex™ workflow. The FirePlex™ assay is carried out in standard 96-well filter plates, with all steps carried out at 37°C or room temperature. After capture and labeling of microRNA targets, assay readout is performed using a standard flow cytometer. Data files from the cytometers are interpreted in FireCode software for analysis and export.

Traditionally, in order to profile microRNAs in complex samples such as cell lysate, plasma, or serum, it is necessary to purify RNA. This step is non-ideal because it often requires laborious protocols, the use of toxic chemicals, and may lead to biases in target recovery. In order to overcome this limitation, we have developed a post-hybridization labeling method for the detection of microRNA targets directly from crude samples, regardless of purity (Figure 2). Additionally, bound targets can be labeled on either the 3' or 5' end in order to discriminate mature from precursor species.

Unlike other labeling schemes, the miRSelect assay labels targets after they have been captured by microRNA-specific probes embedded in the hydrogel particles. Probes are designed to have two binding sites: one for a specific microRNA and another for a universal adapter sequence used for labeling (Figure 2, left). When a microRNA target is captured on its corresponding probe, a universal adapter is attached to that microRNA via ligation. After addition of a reporter species, this binding event is detected via fluorescence. The level of fluorescence is quantitative, providing an accurate indication of target level in a given sample.

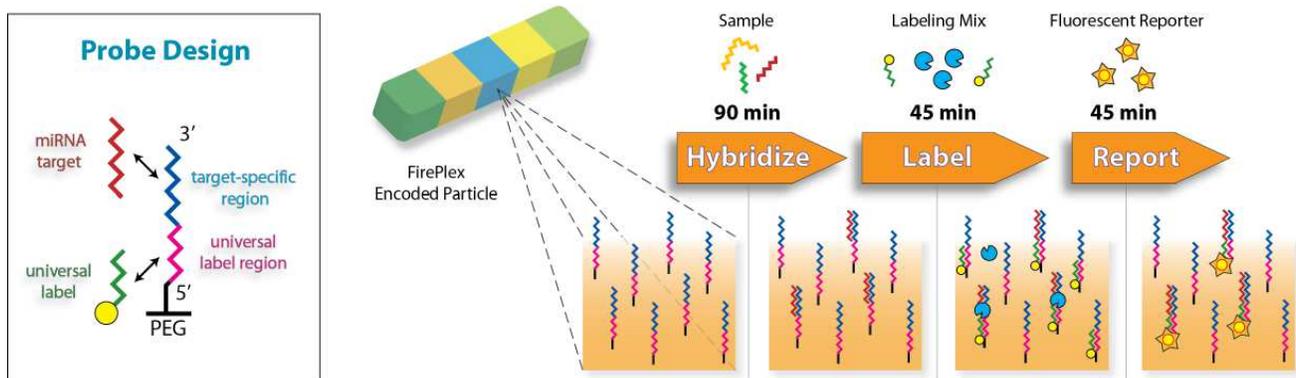


Figure 2: Firefly's post-hybridization labeling scheme. (Left) Probes embedded throughout the particle hydrogel have sites for a specific microRNA and an adjacent site for a universal label, which is detected via fluorescence. (Right) Assay workflow involving hybridization for target capture, attachment of a universal adapter via labeling, and reporting with fluorescence.

Machine to Machine Comparison. To better understand how FirePlex™ miRSelect performs on commercially-available cytometers, we prepared a dilution series assay on five synthetic microRNAs and scanned using three machines including the Millipore Guava® 8HT, BD Accuri™ C6, and Miltenyi MACSQuant®. Data were collected in triplicate over a range from 25 femtomoles (25x10⁻¹⁵ moles) to 100 zeptomoles (100x10⁻²¹ moles). In each case, negative control wells (no target) were used to estimate assay background signals, and those values

were subtracted from the signals at each target level. The results of this comparison are shown in Figure 4.

Even though the three systems tested have slightly different lasers, detectors, filters, and fluidics, the results of the FirePlex assay are virtually identical. Each system shows a broad linear dynamic range over ~5 logs with detection limits of ~1 attomole or less for most targets. In addition, the coefficients of variation for the technical replicates are all ~5% or lower for target levels above 6 attomoles.

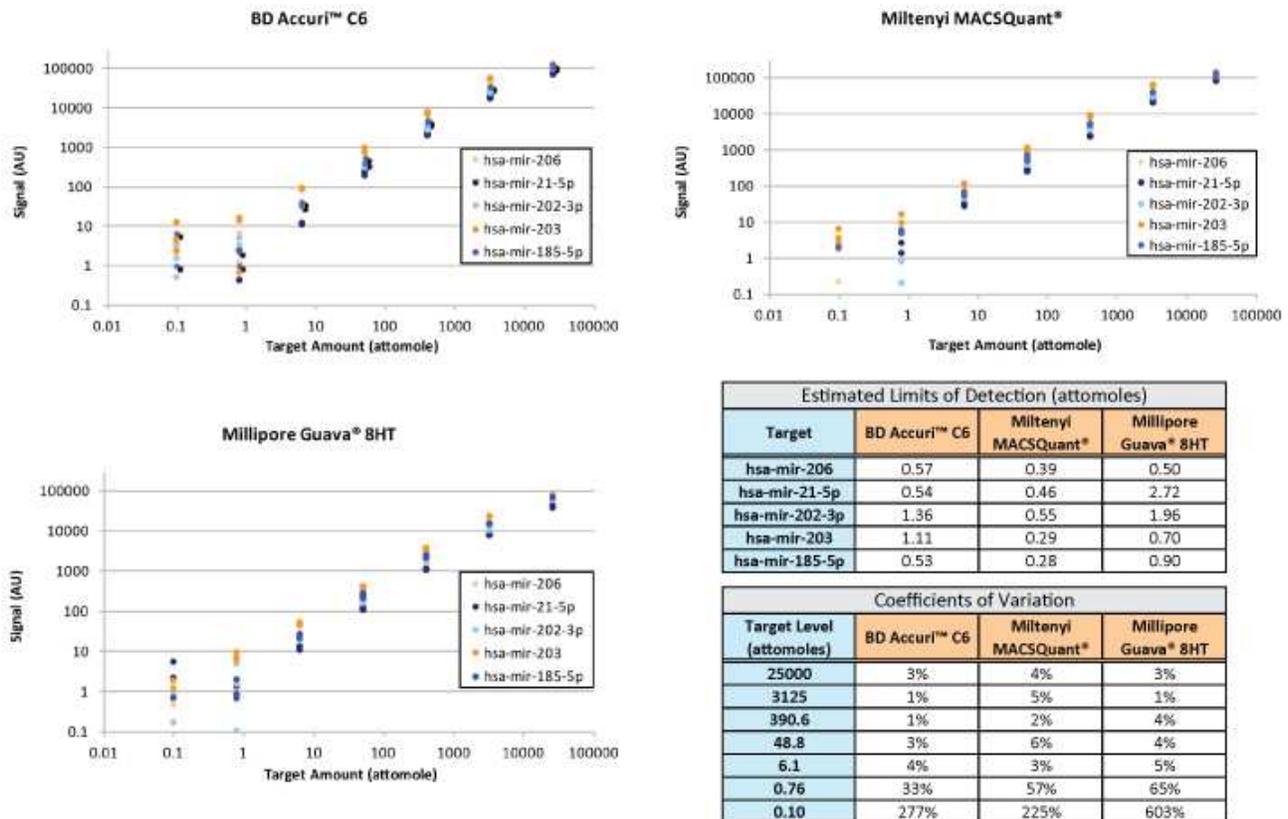


Figure 4: Comparison of FirePlex performance across three commercially-available flow cytometers. Dilution series run in triplicate were analyzed across the three machines and data were analyzed to estimate the limit of detection for each target on each system. Also shown are the average coefficients of variation across the triplicate measurements for each target level.

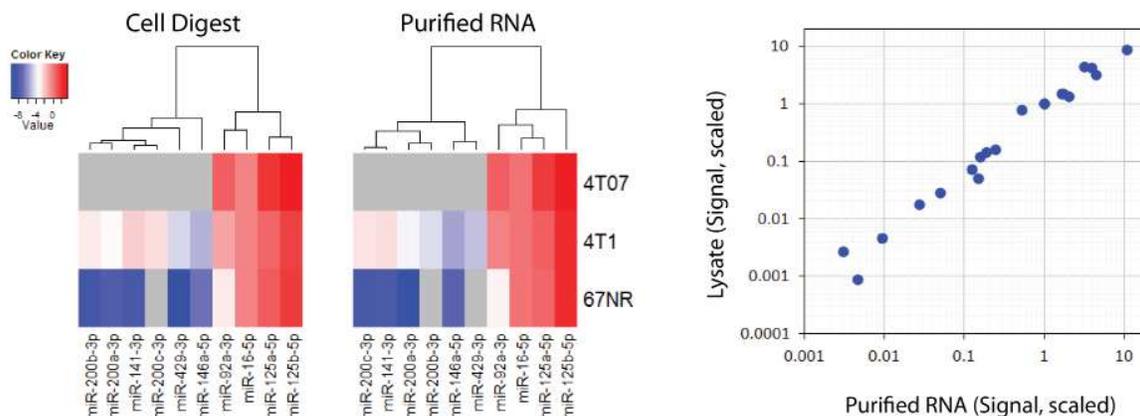


Figure 5: Comparison of microRNA profiling from purified RNA and cell lysates across three cell lines. Shown are heatmaps with \log_2 , miR-16 normalized data. Gray cells represent targets that were not detected. Shown on the right is a scatter plot comparing miR-16 normalized data across the two data sets.

Demonstration of microRNA Profiling in Cell Digests.

We performed expression profiling across three breast cancer cell lines and compared results obtained using purified RNA with those obtained using crude cell digest as the input material. For purified RNA samples, TRIzol[®] was used with the recommended protocol. Because samples were abundant, approximately 2 μg of total RNA was used in the assay. For lysate samples, cells were incubated for 60 minutes at 55°C in Firefly Digest Buffer, after which samples were filtered and 25 μl was added directly into the assay. The cell equivalent for lysate experiments was approximately 10,000 cells. After analysis, data were normalized by miR-16 for comparison. As shown in Figure 5, the profiling data for purified RNA

closely matches those for the crude cell digests. This simple protocol enables high-throughput analysis of cells without the need for toxic chemicals or laborious phase separations.

Conclusion. The FirePlex[™] miRSelect assay provides a high-throughput means to profile microRNA in any species across a broad range of starting material. The multiplex assay is completely customizable for detection of up to 68 targets per sample with a rapid workflow. The assay shows great consistency of performance, regardless of the cytometer used for readout. In all cases, detection limits were near or lower than one attomole and the assay showed linearity over 5 logs of dynamic range.

Related Publications

1. Chapin, SC, Pregibon, DC, and Doyle, PS. [Rapid microRNA Profiling on Encoded Gel Microparticles](#), *Angew. Chem. Int. Ed.*, 50, 2289-2293 (2011).
2. Chapin, S, Pregibon, DC, and Doyle, PS. [High-throughput Flow Alignment of Barcoded Hydrogel Microparticles](#). *Lab Chip* 9:3100-9 (2009).
3. Pregibon DC, Doyle PS. [Optimization of Encoded Hydrogel Particles for Nucleic Acid Quantification](#). *Analytical Chemistry*, 81:4873-81 (2009).
4. Pregibon DC, Toner M, Doyle PS. [Multifunctional Encoded Particles for High-Throughput Biomolecule Analysis](#). *Science*, 315:1393 – 1396 (2007).
5. Dendukuri D, Pregibon DC, Collins J, Hatton TA, Doyle PS. [Continuous Flow Lithography for High-Throughput Microparticle Synthesis](#). *Nature Materials*, 5:365 – 369 (2006).