Multiplex Profiling of Circulating miRNAs for Biomarker Discovery and Verification using the FirePlex® Platform

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MicroRNAs (miRNAs) are both important biomarkers and potential therapeutic targets for a variety of disease areas. Existing technologies for quantifying miRNAs require cumbersome preparation of RNA samples, limiting their sensitivity and clinical utility. Here we demonstrate the FirePlex® miRNA assays, which utilize barcoded hydrogel FirePlex particles that enable the profiling of 5-400 miRNAs directly from biofluids including plasma, serum, urine, exosomes, and tissue sections, as well as from FFPE tissue samples. miRNAs can be scanned on standard flow cytometers and integrated bioinformatics tools are provided to streamline the analysis workflow to minutes rather than days. This technology platform is ideally suited for miRNA biomarker discovery and verification studies from limited sample inputs.

Introduction

Our multiplex miRNA assays provide a sample-to-results solution for miRNA profiling and quantitation, combining a simple workflow that eliminates separate RNA extraction and cDNA synthesis. The integrated FirePlex Analysis Workbench software is capable rapid data analysis and generating publication-ready figures within minutes.

FirePlex particle technology has key properties that enable enhanced performance over existing bead-based systems. The hydrogel offers greater surface area, significantly improving hybridization signal robustness. Particles are biologically inert, enabling their use with complex sample types without aggregating and compromising function.

Each particle contains a single sequence miRNA probe unique barcode, allowing multiplexing of up to 65 miRNAs per well. An average of 20 particles are used to detect signal for each miRNA (equivalent to 20 individual spot measurements of a microarray) to provide a high level of signal robustness.

The FirePlex miRNA assay protocol (Figure 1) is simple and can be performed simultaneously across 96 wells, generating 6,528 data points in each run. The assay consists of (a) capture of selected miRNAs on encoded hydrogel particles, (b) ligation of specific adapters to each bound miRNA, (c) target amplification using a single, universal primer and (d) recapture of amplified targets onto the hydrogel encoded hydrogel particles.

This method eliminates the need for extensive RNA purification, reverse transcription or probe amplification, while minimizing primer-dependent interactions that can lead to amplification bias seen in most PCR assays. Assay readout is performed using a standard flow cytometer. The FCS fluorescence intensity data is decoded into target concentration in each well, and the data can be quickly visualized, manipulated and compared using the FirePlex® Analysis Workbench software suite.

FirePlex platform comparison vs existing technologies

Reference RNA from three tissue types was profiled to benchmark the FirePlex miRNA assay against existing profiling methods including TaqMan Low Density Array (TLDA) qPCR assays and Illumina TruSeq sequencing (Figure 2A). In each case, FirePlex miRNA assays showed excellent correlation (Pearson >0.92). In an independent study, similar results were observed when comparing FirePlex to the TruSeq system (data not shown).

We also assessed the specificity of the FirePlex miRNA assay using closely-related miRNAs (Figure 2B). Low cross-reactivity was observed for all off-target probes, typically 2-8%.

Robust miRNA detection from low biofluid inputs

To demonstrate the high sensitivity of the FirePlex miRNA Assay, we measured the number of miRNAs detected from varying input amounts of pooled human serum (Figure 3). We robustly detect most targets from the 48-plex panel in as little as 100 µL of serum. To demonstrate the breadth of targets detected, we profiled four samples across seven partially-overlapping 65-plex panels (402 unique probes total) and detected the majority of miRNAs above the limit of detection.

miRNA profiling from complex sample types

Independent replicates from six human biofluid sample types, and FFPE tissues were profiled using the FirePlex miRNA assay, and miRNA profiles were compared to reference RNA (RNA pooled from human brain, lung, and liver tissue). Robust profiles were obtained across all sample types tested, including hemoplastic plasma, demonstrating the compatibility of this assay with a broad range of sample types (Figure 4A).

In addition, the FirePlex miRNA assay yields highly reproducible data directly from biofluid samples (Pearson >0.96, Figure 5A).

Integrated software solution

Proper interpretation of profiling data is a critical component of biomarker development. The FirePlex Analysis Workbench (Figure 6) provides a means to decode the particles and transform the miRNA profiles into publication-quality figures in minutes.

The software allows interpretation of multiple experiments simultaneously, normalizes data, and simplifies advanced statistical analysis.

Figure 1: Multiplex RNA Assay workflow. After capture, labelling and amplification of target miRNAs, assay readout is performed using a standard flow cytometer. Data Box can be interpreted with the FirePlex Analysis Workbench software for analysis and export.

Figure 2. A. Reference tissue type. B. Assay reproducibility was assessed for replicate human serum, plasma, and reference RNA samples, respectively. Figure 3. Robust miRNA detection from low biofluid inputs. Figure 4. A. Number of target miRNAs detected above background using a 48-plex panel in pooled human serum. B. Number of target miRNAs detected above background in pooled human serum, pooled human plasma, exosomes isolated from pooled serum and pooled human RNA (serum only and liver profiled detecting 402 unique targets).

Figure 5. Three independent samples of human plasma containing EDTA, heparinized plasma, serum, semicocytic fluid, exosomes isolated from urine, saliva, and FFP were profiled using FirePlex® miRNA profiling. Heatmap demonstrates the miRNA signature for each sample type. B. Assay reproducibility was assessed for replicate human serum, plasma, and reference RNA samples, respectively.

Figure 6. An integrated software solution, the FirePlex Analysis Workbench provides sample opportunity for multicolored data analysis, including heat maps, background subtraction, normalization, absolute quantification, and ANOVA data analysis.