Multiplex profiling of circulating miRNAs for biomarker discovery and verification using the FirePlex® platform

M. Tackett, G. Doran, I. Diwan, G. Tejada, C. Rafferty, E. Atabakhsh, and D. Pregibon
Abcam Inc. One Kendall Square, Building 200, Cambridge, MA 02139. FirePlexSales@abcam.com

MicroRNAs (miRNAs) are both important biomarkers and potential therapeutic targets for a variety of disease areas. Existing tec hnologies 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 enables the profiling of 5-400 miRNAs directly from biofluids including plasma, serum, urine, and exoso mes, as well as from FFPE tissues. These particles 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 styrene bead based systems. Porous hydrogel offers greater surface area, significantly improving hybridization detection sensitivity. Particles are biologically inert, enabling their use with complex sample types without aggregating and compromising function.

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

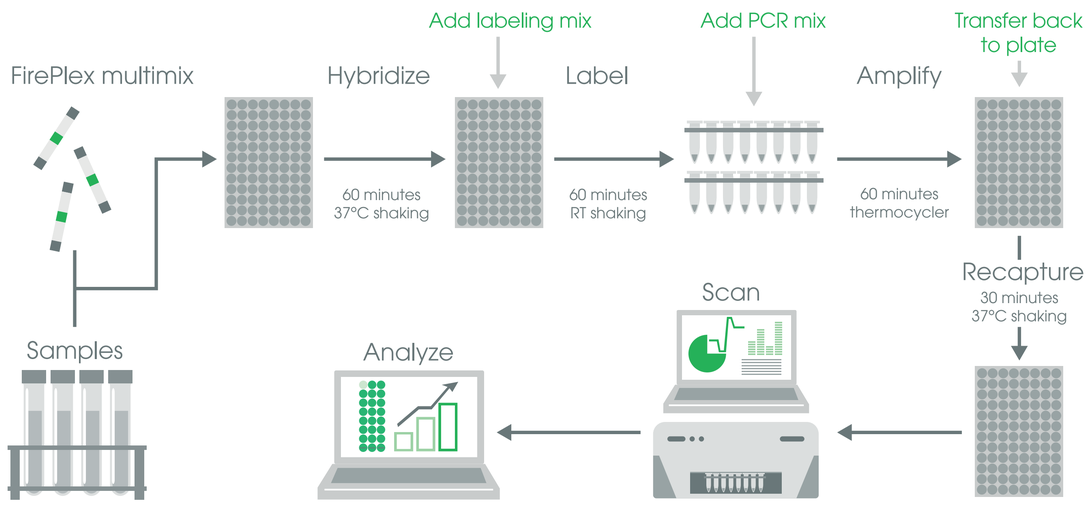


Figure 1. Multiplex miRNA Assay workflow. After capture, labeling and amplification of target miRNAs, assay readout is performed using a standard flow cytometer. Data files are interpreted with the FirePlex Analysis Workbench software for analysis and export.

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 particles.

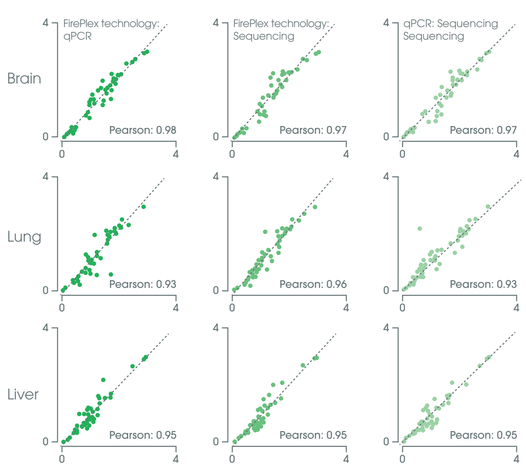
This method eliminates the need for extensive RNA purification, reverse transcription or pre-amplification, while mitigating primer-dependent interactions that can lead to amplification bias seen in most PCR assays. Assay readout is performed using a standard flow cytometer. The FCS fluorescence intensity 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 NanoString 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%.

A



B

	Let-7a	Let-7b	Let-7c	Let-7d	Let-7e	Let-7f	Let-7i
Let-7a	100.00%	8.95%	7.91%	3.91%	5.81%	5.31%	2.83%
Let-7b	5.19%	100.00%	19.52%	2.86%	3.51%	3.24%	3.37%
Let-7c	14.43%	18.64%	100.00%	7.20%	7.92%	6.89%	6.93%
Let-7d	4.63%	3.52%	2.89%	100.00%	2.00%	2.18%	2.08%

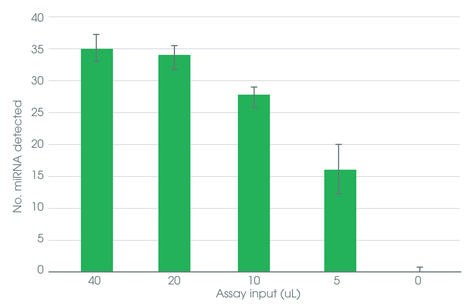
	miR-302a	miR-302b	miR-302c	miR-302d	miR-302h
miR-302a	100.00%	1.95%	1.89%	2.54%	1.26%
miR-302b	5.10%	100.00%	7.86%	6.55%	5.64%
miR-302c	1.52%	2.04%	100.00%	2.64%	0.88%
miR-302d	2.15%	2.30%	2.37%	100.00%	1.32%

Figure 2. A. Reference tissue samples profiled using three technologies. Input amounts of 1 µg, 60 ng and 10 ng were used for Illumina TruSeq, Taqman TLDA and FirePlex, respectively. Data are plotted as fold change vs fold change relative to averaged sample. B. Specificity with 1 attomole of synthetic target (designated by row) spiked into MS2 RNA. Cross-reactivity to related probes (specified by column) measured using the FirePlex miRNA Assay.

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 10 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 majoriy of miRNA above the limit of detection.

A



B

Sample	Targets above detection (n=402)
Plasma	321
RNA	308
Exosomes	276
Serum	258

Figure 3. A. Plots of log2 intensity for FirePlex technology at low total RNA input amounts. B. Pearson correlations for TLDA and FirePlex technologies for each sample amount. C. Coefficient of variation for top 50% and bottom 50% of expressers for 25 pg input amount indicates lower CVs for the FirePlex technology (error bars are standard error, n=30 in each group).

Reproducible expression profiles from low cell numbers

Direct detection of miRNAs from cell lysate greatly simplifies the workflow for high throughput experiments. miRNA signatures are reproducibly detected from 10–1,000 K562 cells sorted by flow cytometry into single wells (Figure 4). These signatures are highly correlated with TRIzol® prepared total RNA profiling from the 100,000 cells of the same cell source (Pearson 0.89, not shown).

A

	1000 cells	100 cells	25 cells
1000 cells	1.00	0.91	0.89
100 cells		1.00	0.97
25 cells			1.00

B

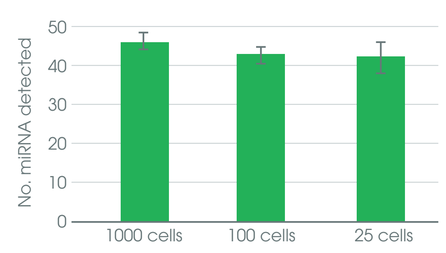


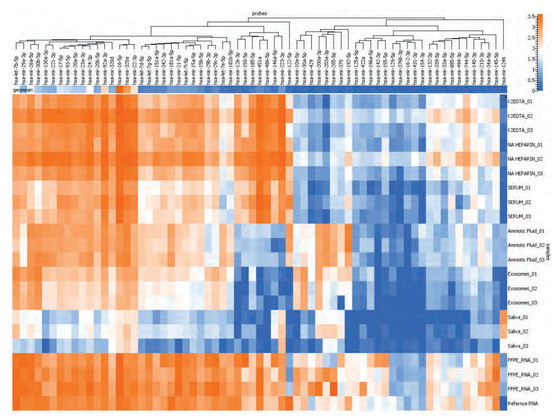
Figure 4. A. Pearson correlations for 48 miRNA measured from 3 input amounts of K562 cells, sorted directly from PBS into FirePlex Digest buffer (1:1) and incubated for 30 min at 55°C prior to FirePlex profiling. B. Number of targets detected in each sample is consistent at each cell input amount.

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 from all sample types tested, including heparinized plasma, demonstrating the compatibility of this assay with a broad range of sample types (Figure 5A).

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

A



B

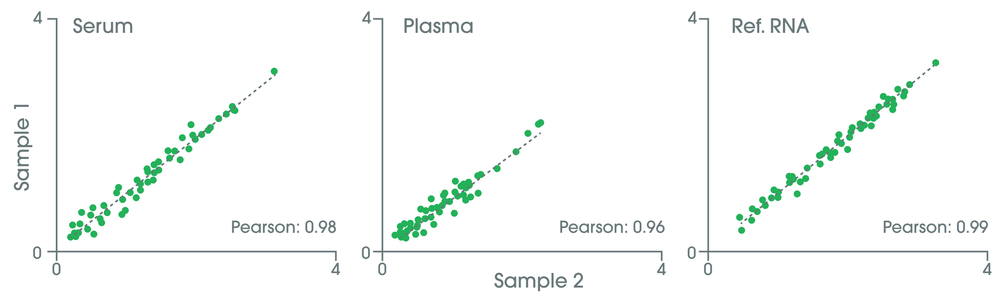


Figure 5. A. Three independent samples of human plasma containing EDTA, heparinized plasma, serum, amniotic fluid, exosomes isolated from urine, saliva, and FFPE tissues were used for FirePlex miRNA profiling. Heatmap demonstrates the miRNA signature for each sample type. B. Assay reproducibility was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

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 cytometry files into publication-quality figures in minutes.

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

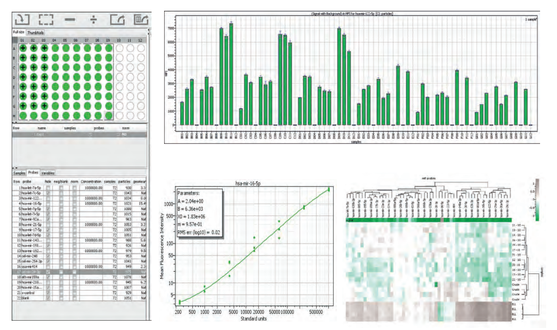


Figure 6. An integrated software solution, the FirePlex Analysis Workbench provides ample opportunity for multifaceted data analysis, including heat maps, background subtraction, normalization, absolute quantification, and ANOVA data analysis.