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FirePlex® miRNA Assay

Multiplex microRNA profiling from
low sample inputs

Abstract

We introduce a new assay for multiplex microRNA (miRNA) discovery and verification that enables simultaneous profiling of up to 65 miRNAs directly from biofluids, with no need for RNA purification. The platform is built on porous hydrogel particles that maximize miRNA capture capacity while being tolerant to factors that commonly foul other platforms, such as serum albumin, complex glycoproteins, heme compounds and heparin. We demonstrate miRNA profiling with PCR sensitivity from only 10 µL of crude biofluids or 100 pg of purified RNA, and introduce easy-to-use bioinformatics tools that are integrated with the assay software for fast and efficient data analysis.

Overview

miRNA profiling has tremendous potential for aiding diagnosis and determining prognosis of a broad range of diseases. Numerous studies have demonstrated that miRNAs are stable in fresh blood samples and samples frozen long-term[1], making miRNAs ideal biomarkers for prospective and retrospective studies. However, several technical challenges must be overcome when using circulating miRNAs as biomarkers. These include low abundance of miRNAs in biofluids, contaminants introduced during sample preparation and low starting volumes of precious biofluid.

We have developed the FirePlex® miRNA Assay to overcome these challenges and allow biomarker discovery and verification studies directly from biofluids, without need for RNA purification or pre-amplification.
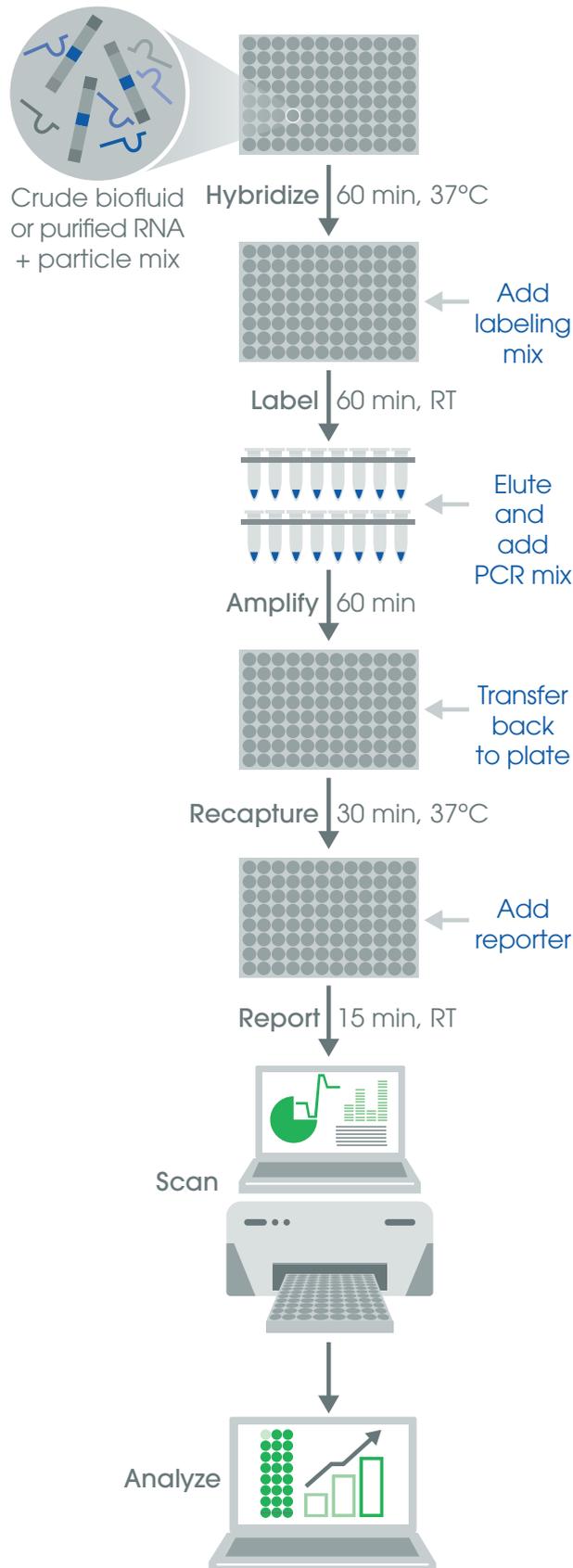
miRNA detection direct from biofluids and FFPE

With the FirePlex miRNA Assay robust miRNA profiles can be obtained directly from many sample types, including serum, plasma, crude exosomes, cell suspensions, urine, saliva, and FFPEs. Most protocols for isolating RNA from plasma and serum require relatively large sample volume (200 µL) and some enrichment methods can have profound effects on miRNA profiles[2]. Measurement directly from biofluids using the FirePlex miRNA Assay reduces such sample bias, while also lowering required sample input and reducing running time.

Designed for discovery and verification studies

The FirePlex miRNA Assay is a customizable biomarker development platform, designed for both discovery and verification studies. Researchers can select between pre-designed panels for key research areas, larger discovery panels, or design custom panels to detect any miRNA from any species, whether annotated in miRBase or not. Assays are read out using a standard flow cytometer.

Assay workflow

Crude biofluid or purified RNA + particle mix

Hybridize | 60 min, 37°C

← **Add labeling mix**

Label | 60 min, RT

← **Elute and add PCR mix**

Amplify | 60 min

← **Transfer back to plate**

Recapture | 30 min, 37°C

← **Add reporter**

Report | 15 min, RT

Scan

Analyze

Protocol

The FirePlex miRNA Assay is performed in a 96-well filter plate. The workflow involves six main steps:

1. Incubation of biofluid with digest buffer (45 minutes, not required if starting from purified RNA).

2. Addition of FirePlex particles, hybridization buffer and sample to assay wells, followed by hybridization step (60 minutes).

3. Incubation with labeling buffer (60 minutes).

4. Elution of miRNAs from particles, followed by PCR amplification (60 minutes).

5. Transfer back to plate, followed by recapture (30 minutes).

6. Incubation with a fluorescent reporter (15 minutes) and scanning on a standard flow cytometer.

Overall, the assay takes 4–7 hours from sample to data, depending on how many samples are processed in parallel and which cytometer is used for scanning.

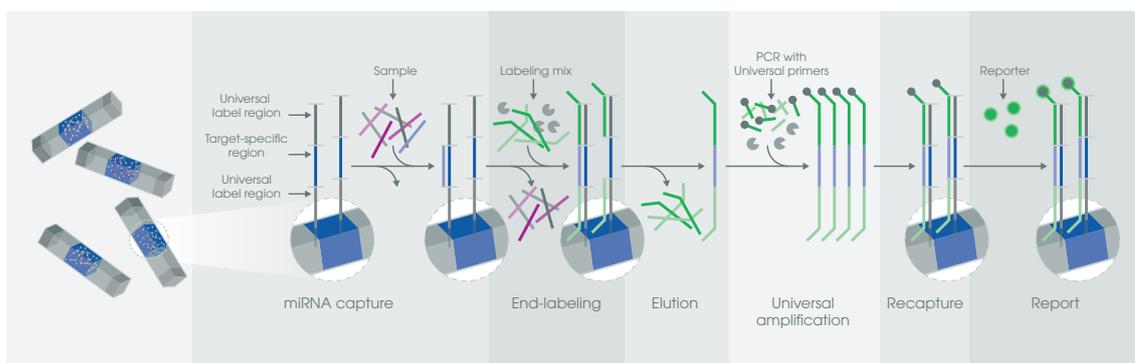**Figure 1. FirePlex miRNA Assay workflow.**
After capture, labeling and amplification of target miRNAs, assay readout is performed using a standard flow cytometer. Data files from the cytometers are interpreted with the FirePlex Analysis Workbench software for analysis and export.

Molecular workflow

miRNAs are captured by miRNA-specific probes embedded in the three-dimensional volume of FirePlex polyethylene glycol hydrogel particles. miRNA-specific probes have three binding sites: one for the miRNA and two for universal adapter sequences used for amplification. After miRNAs are captured, universal adapters are ligated to the miRNAs and amplified by PCR with labeled primers (Figure 2).

Importantly, miRNA capture also acts as a purification step, depleting PCR inhibitors, including heparin, that reduce sensitivity in other detection systems. This makes the assay ideal for the detection of miRNA targets directly from biofluid or low quality RNA samples regardless of purity.

Following PCR amplification, miRNAs are re-hybridized to the probes and detected via fluorescence. The level of fluorescence is quantitative, providing an accurate indication of target level in a given sample.

Figure 2. Molecular workflow for the FirePlex miRNA Assay.
Probes embedded throughout the particle hydrogel have sites for a specific miRNA and two adjacent sites for universal amplification. The assay workflow involves direct miRNA capture, end-labeling with adapters followed by universal amplification, recapture and reporting with fluorescence.

Flexible and customizable panel offering

To meet the demands of miRNA biomarker discovery and verification, we provide panels to support each stage of miRNA biomarker development.

For discovery

Pre-designed discovery panel contain 400 circulating miRNAs identified from peer-reviewed studies. Our discovery panels can be customized for rodent, immunology or plasma/serum markers.

For verification

Focus panels contain 65 miRNA probes specific for research areas such as liver toxicity, oncology, immunology, cardiology and neurology. Alternatively, you can design your own custom panel consisting of 5–65 miRNAs, for any species whether they're listed in miRBase or not.

See our panel offering:
www.abcam.com/FirePlexmiRNAPanels

High detection sensitivity and specificity

Sensitivity

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 or pooled RNA from human brain, lung and liver samples (Figure 3). As shown, we robustly detect most targets from the 48-plex panel in as little as 10 µL of serum or 100 pg of RNA.
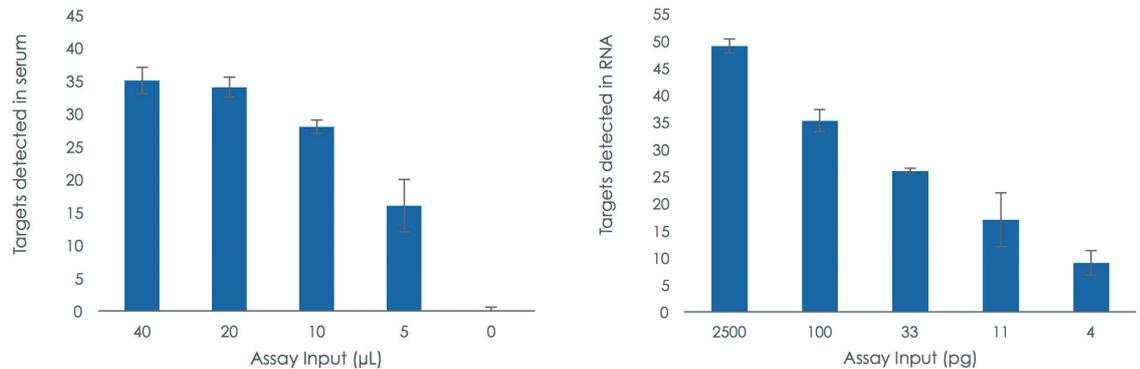

Figure 3. Sensitivity and linearity of the FirePlex miRNA Assay.
Number of target miRNAs detected above background using a 48-plex panel in pooled human serum (left) and pooled human brain, lung and liver RNA samples (right). The detection limit is calculated as the sum of 3 standard deviations and the mean value of 3 off-species control probes.

Assay linearity across serial dilutions

To assess assay linearity, we performed serial dilutions of purified pooled RNA from human brain, lung and liver tissue, and plotted the normalized signal for each miRNA against total RNA input (Figure 4). The results demonstrate good signal linearity across multiple targets and RNA input amounts.
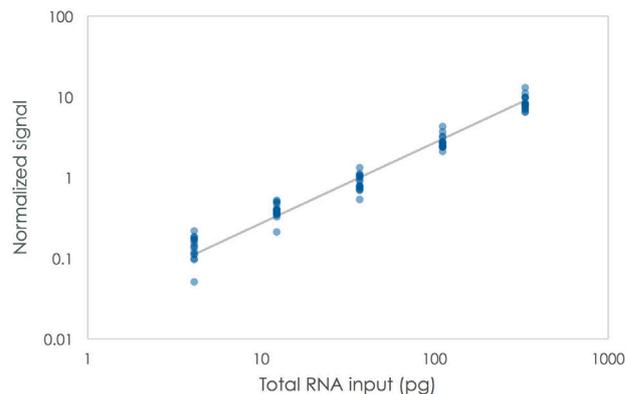
Figure 4.
Three-fold serial dilutions of total RNA purified and pooled from brain, lung, and liver tissue. Data points represent individual targets detected at each dilution. Only targets detected at the top four dilutions (16 in total) were included in this analysis and all data were normalized for display in a common range. The solid line indicates expected signal.

Assay specificity

We also assessed specificity of the assay using miRNAs let-7a, 7b, 7c and 7d individually spiked into a panel containing probes for 7a, 7b, 7c, 7d, 7e, 7f, 7g and 7i, which differ by one or two nucleotides (Figure 5, top). We observed low cross-reactivity for all off-target probes, typically 2–8%. We obtained similar results for the miR-302 family (Figure 5, bottom).

	Let-7a-5p	Let-7b-5p	Let-7c-5p	Let-7d-5p	Let-7e-5p	Let-7f-5p	Let-7g-5p	Let-7i-5p
Let-7a-5p	100.00%	4.0%	12.0%	5.5%	7.9%	7.1%	2.7%	1.6%
Let-7b-5p	1.3%	100.00%	23.1%	0.7%	0.5%	0.5%	0.6%	0.7%
Let-7c-5p	14.435.6	19.1%	100.00%	2.0%	2.1%	1.8%	1.7%	1.7%
Let-7d-5p	1.6%	1.3%	1.4%	100.00%	1.1%	1.0%	1.0%	0.8%

miRNA	Sequence
hsa-let-7a-5p	UGAGGUAGUAGGUUGUAUAGUU
hsa-let-7b-5p	UGAGGUAGUAGGUUGUGUGGUU
hsa-let-7c-5p	UGAGGUAGUAGGUUGUAUGGUU
hsa-let-7d-5p	AGAGGUAGUAGGUUGCAUAGUU
hsa-let-7e-5p	UGAGGUAGGAGGUUGUAUAGUU
hsa-let-7f-5p	UGAGGUAGUAGAUUGUAUAGUU
hsa-let-7g-5p	UGAGGUAGUAGUUUGUACAGUU
hsa-let-7i-5p	UGAGGUAGUAGUUUGUGCUGUU

	miR-302a-3p	miR-302b-3p	miR-302c-3p	miR-302d-3p	miR-302h-3p	miR-106a-5p
miR-302a-3p	100.00%	2.3%	1.8%	1.8%	0.6%	0.4%
miR-302b-3p	1.8%	100.00%	2.9%	1.4%	0.5%	0.5%
miR-302c-3p	3.4%	4.3%	100.00%	4.2%	0.7%	0.5%
miR-302d-3p	2.99%	2.1%	2.8%	100.00%	0.8%	0.4%

| miRNA	Sequence
hsa-miR-302a-3p	UAAGUGCUUCCAUGUUUUGGUGA
hsa-miR-302b-3p	UAAGUGCUUCCAUGUUUUAGUAG
hsa-miR-302c-3p	UAAGUGCUUCCAUGUUUCAGUGG
hsa-miR-302d-3p	UAAGUGCUUCCAUGUUUGAGUGU
hsa-miR-302h-3p	ACAAAGUGCUUCCCU-UUAGAGU--
hsa-miR-520h-3p	AAAAGUGCUUACAGUGCA-GGUAG

Figure 5. Assay specificity for families of highly similar miRNAs.
(Top left) let-7a, 7b, 7c and 7d were spiked into a panel containing probes for 7a, 7b, 7c, 7d, 7e, 7f, 7g and 7i, (top right) sequences for the let family of miRNAs, with sequence differences highlighted in red. (Bottom left) miR-302a, miR-302b, miR-302c and miR-302d were spiked into a panel containing miR-302a, miR-302b, miR-302c, miR-302d, miR-302h and miR-106a, (bottom right) sequences for the miR-30 family, with sequence differences highlighted in red.

Integrated bioinformatics tools for miRNA discovery and analysis

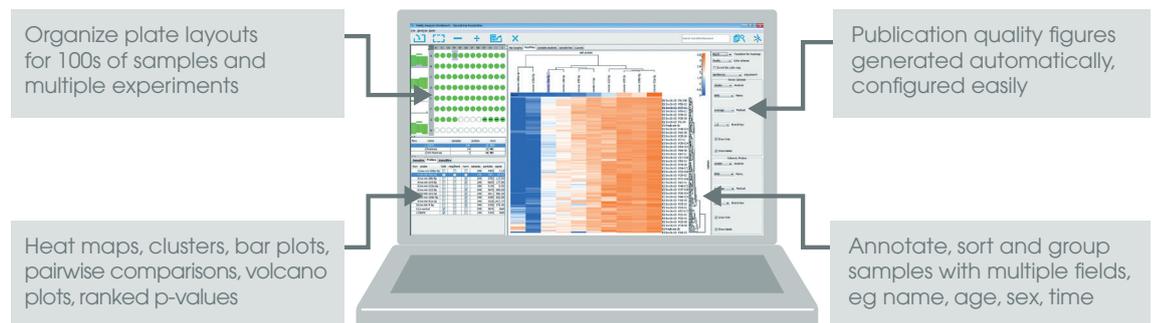## FirePlex Discovery Engine: identify relevant miRNAs for any topic

Designing a custom panel for miRNA discovery or verification requires initial identification of key miRNAs in that research area. The FirePlex Discovery Engine searches peer-reviewed publications to find a list of miRNAs relevant to the topic, and using natural language processing, ranks them according to relevance, occurrences and journal impact. The Discovery Engine also provides a list of genes that were also referenced in those publications.
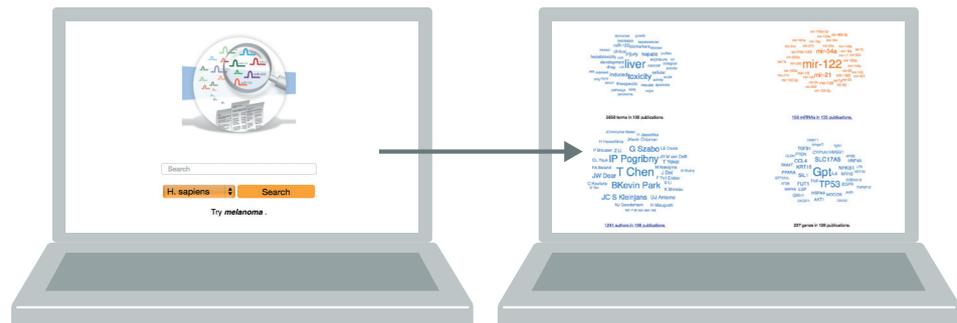
Figure 6.
miRNA identification with the FirePlex Discovery Engine. The Discovery Engine searches the scientific literature and identifies the most relevant miRNAs for any research topic and species.

The FirePlex Analysis Workbench software

Proper interpretation of profiling data is a critical component of biomarker development. The FirePlex Analysis Workbench 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.

Organize plate layouts for 100s of samples and multiple experiments

Publication quality figures generated automatically, configured easily

Heat maps, clusters, bar plots, pairwise comparisons, volcano plots, ranked p-values

Annotate, sort and group samples with multiple fields, eg name, age, sex, time

Figure 7. The FirePlex Analysis Workbench.
The software features tools for organizing and annotating data. Point and click GUI, no scripts, Java-based, runs everywhere, normalization and import of .fcs files.

Conclusions

The FirePlex miRNA Assay provides a flexible and robust method that enables profiling miRNAs in sample types that have previously presented challenges for researchers. The assay is sensitive, specific, and allows high-throughput analysis required for biomarker discovery studies. The use of biofluid digests eliminates a major source of pre-analytical variability while minimizing RNA processing workflow. In addition, the FirePlex Analysis Workbench provides a largely automated means to rapidly visualize and interpret experimental data.

References

1. Farina NH, Wood ME, Perrapato SD, Francklyn CS, Stein GS, Stein JL, Lian JB. Standardizing analysis of circulating miRNA: clinical and biological relevance. *J Cell Biochem*, **115**, 805–811 (2014).

2. Kim Y-K, Yeo J, Kim B, Ha M, Kim VN (2012). Short structured RNAs with low GC content are selectively lost during extraction from a small number of cells. *Mol Cell*, **46**, 893–895 (2012).