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# Development of an extractionless, multiplexed assay for the direct detection of miRNAs in liver tissue


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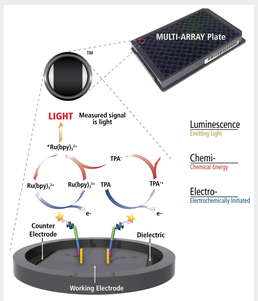
## PURPOSE

MicroRNA (miRNA) detection has become a powerful tool to understand disease pathology, which has led to an increasing interest in the use of miRNAs as molecular biomarkers. Certain miRNAs, including miR-122, are abundantly expressed in the liver and are considered biomarkers for hepatotoxicity. However, miRNA quantification is almost exclusively conducted through miRNA sequencing or singleplex measurements that require amplification. We developed a panel of convenient multiplexed assays that can directly measure miRNAs associated with liver damage across multiple species.

## METHODS

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.




**Electrochemiluminescence Technology**

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Surface coatings can be customized.

**Basis of the N-PLEX platform**

N-PLEX® plates contain 10 unique capture oligonucleotides that are bound to their corresponding spots on the electrode surface. Detection of a nucleic acid sequence is accomplished by hybridization of one or more probes with sequence complementary to these capture oligos and the nucleic acid of interest, followed by detection via electrochemiluminescence (i.e. biotin/streptavidin SULFO-TAG interactions). Blocking, hybridization, and detection are completed using proprietary MSD® buffers and diluents.

**Rodent tissue samples**

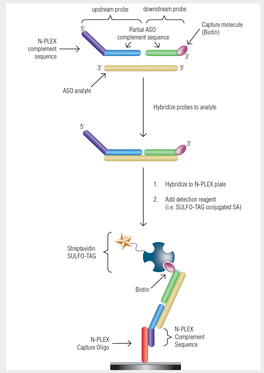
Liver, kidney, heart, brain and lung tissues were harvested from mice and rats, homogenized in Nucleic Acid Purification Lysis Solution, and lysates were collected.

**Human cell line samples**

Human hepatocytes (HepaRG cell line) were cultured under manufacturer's suggested conditions in William's E Medium with Thaw/General Medium Supplement. Pellets were collected at 4 hours post-incubation, then homogenized in Nucleic Acid Purification Lysis Solution, and lysates were collected.

**miRNA detection via two-probe approach**

The two-probe detection assay used probes that were complementary to the nucleotides of one-half of the miRNA. The upstream probe contained a spot-specific sequence at the 5′ end that allowed for hybridization to the N-PLEX plates. The use of spot-specific sequences enables detection of up to 10 distinct miRNAs per well when multiplexed. The downstream probe contained a biotin on the 3′ end for detection. The probes were hybridized to the miRNA and then to spot-specific capture oligos on the N-PLEX plates. SULFO-TAG™ labeled streptavidin was then used to detect the captured miRNA.



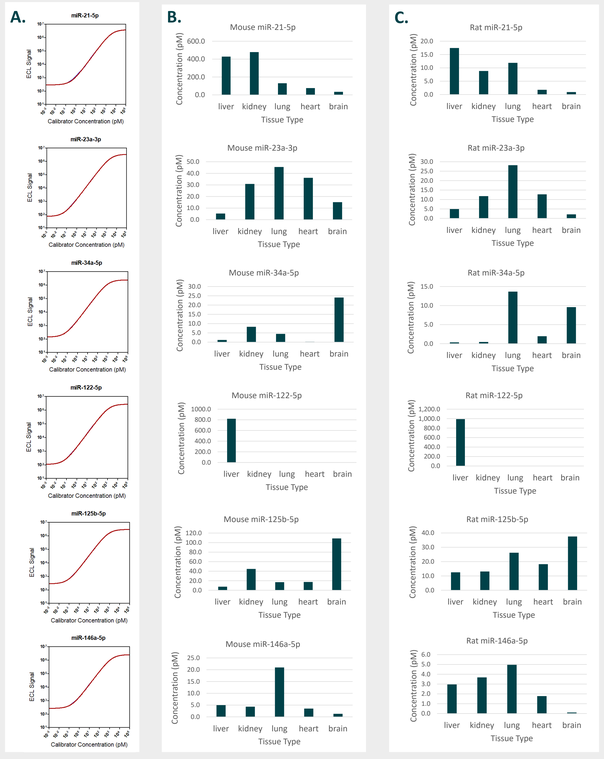

## RESULTS

### LLOD and Hill Slope

| miRNA | Multiplex | | Singleplex | |
|---|---|---|---|---|
| | LLOD (fM) | Hill Slope | LLOD (fM) | Hill Slope |
| miR-15a-5p | 40.2 | 0.97 | 42.2 | 1.03 |
| miR-21-5p | 66.3 | 0.97 | 43.3 | 1.01 |
| miR-23a-3p | 48.0 | 0.98 | 71.0 | 1.01 |
| miR-34a-5p | 30.6 | 1.00 | 48.2 | 1.02 |
| miR-122-5p | 37.8 | 1.00 | 41.5 | 1.03 |
| miR-125b-5p | 27.0 | 0.98 | 17.0 | 0.99 |
| miR-146a-5p | 40.2 | 0.97 | 57.3 | 0.97 |
| miR-148a-3p | 21.2 | 0.97 | 27.4 | 0.98 |
| miR-192-5p | 37.3 | 0.98 | 43.8 | 1.03 |
| miR-193a-3p | 58.3 | 0.99 | 50.1 | 1.02 |
| miR-194-5p | 70.6 | 1.02 | 53.6 | 1.03 |
| miR-210-3p | 91.2 | 0.99 | 53.7 | 1.01 |
| miR-221-3p | 24.5 | 0.98 | 33.4 | 0.99 |

Table 1. Probes were designed against several liver damage miRNA biomarkers and were run in singleplex and multiplex probe systems. Calibration curves were run using synthetic miRNA templates, and the lower limit of detection (LLOD) was determined. The optimized assays were able to specifically identify target miRNAs with a LLOD of lower than 100 fM in both singleplex and multiplex.

### Multiplexed Testing for Mouse and Rat Tissues




## RESULTS

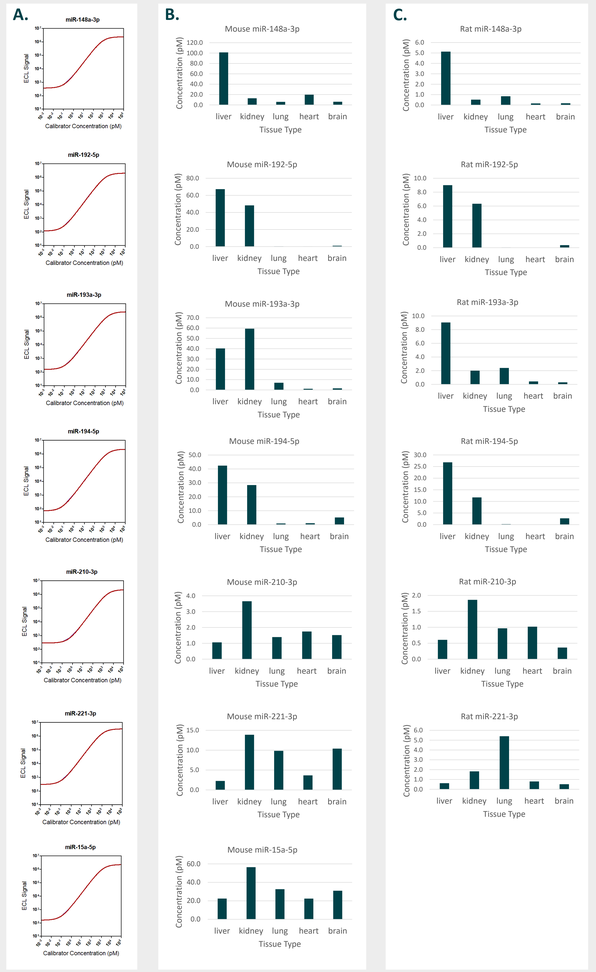



Figure 1A. Calibration curves were generated using synthetic RNA templates.

Figure 1B and 1C. Lysed tisssue samples from mouse (B) and rat (C) were generated as follows: tissue samples were homogenized, spun down, and supernatants were collected. Twenty microliters of the lysed sample was used in the assay without further extraction. A multiplexed mixture of the upstream and downstream probes were hybridized to their corresponding miRNAs in sample, allowing detection of up to 10 distinct miRNAs per well. It was shown that the miRNAs could be directly quantified from various tissue lysates without any extraction steps or amplification. Note: The rat assay for miR-15a-5p is still under development.

## RESULTS

### Multiplexed Testing for HepaRG Human Cell Line

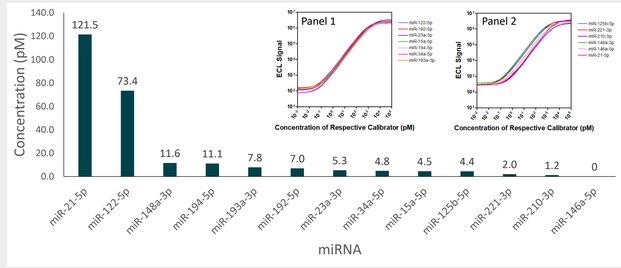



Figure 2. HepaRG human hepatocyte cells were cultured and cells were lysed and spun down. Supernatants were collected and 20 µL of sample was used in the assay without further extraction. Multiplexed probes were hybridized to target miRNAs. We detected all but miR-146a in lysates from the hepatocyte cell line.

### Specificity Testing against Synthetic Targets

| Target Sequence ECL Signal | Similar Sequence ECL Signal | % Signal | # of Conserved Bases |
|---|---|---|---|
| miR-15a-5p | miR-15b-5p | | |
| 840648 | 86 | 0.00% | 18/22 (82%) |
| miR-21-5p | miR-21b | | |
| 462911 | 84 | 0.00% | 18/22 (82%) |
| miR-23a-3p | miR-23b-3p | | |
| 724221 | 16458 | 2.30% | 20/21 (95%) |
| miR-122-5p | miR-122b | | |
| 372137 | 121 | 0.03% | 18/22 (82%) |
| miR-146a-5p | miR-146b-5p | | |
| 471224 | 42 | 0.00% | 20/22 (91%) |

| Target Sequence ECL Signal | Similar Sequence ECL Signal | % Signal | # of Conserved Bases |
|---|---|---|---|
| miR-148a-3p | miR-148b-3p | | |
| 374496 | 118.5 | 0.00% | 20/22 (91%) |
| miR-192-5p | miR-215-5p | | |
| 947428 | 5118 | 0.50% | 19/21 (90%) |
| miR-193a-3p | miR-193b-3p | | |
| 1185488 | 5022 | 0.40% | 19/22 (86%) |
| miR-221-3p | miR-4288 | | |
| 1211579 | 12 | 0.00% | 15/23 (65%) |

Table 2. miRNAs that have similar sequences to the target miRNAs of interest were identified using BLAST. Each of the nine similar miRNAs were produced synthetically and tested on the N-PLEX assays in singleplex to evaluate specificity. ECL signals from the similar miRNAs were < 2.5% of the target signal (both were tested at 800 pM).

## CONCLUSION

We demonstrated sensitive, multiplexed measurement of liver damage-associated biomarker miRNAs in a human cell line and rodent tissue samples. These assays do not require RNA extraction and do not use a polymerase-based detection system, allowing simple workflows and a time-to-result of three to four hours. In a one-plate run, up to 10 miRNAs can be measured in 40 samples when run in duplicate, or 80 samples when run in singlicate, providing a method that is both quantitative for miRNA and amenable to high-throughput applications.

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