miR-Direct®: A Sensitive and Specific Method for Quantification of miRNAs Directly from Plasma and other Biofluids

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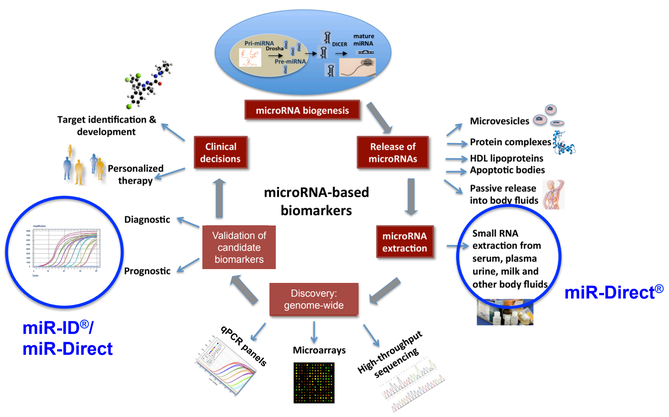
Abstract

Circulating microRNAs (miRNAs) have great potential as biomarkers, but current methods for their accurate quantification are impeded by inefficient and inconsistent miRNA purification from biofluids, the very low concentrations of miRNAs in biofluids, and the difficulty in eliminating inhibitors of PCR reactions that co-purify with RNA. We have developed a novel method, called miR-Direct® for purification, concentration, and quantification of miRNAs from biofluid samples by RT-qPCR while avoiding procedures such as organic extraction, ethanol precipitation, and column adsorption/elution procedures that are known to produce inconsistencies in miRNA quantification. Samples are first treated to release miRNAs from their protein and lipid-containing complexes and vesicles while protecting them from degradation by RNases. A spike-in miRNA is added to serve as a positive control and a marker of experimental variability. Next, miRNAs of interest are hybridized in multiplex with miRNA-specific probes in solution (targeted capture). These hybridized complexes are then captured and concentrated on magnetic beads and stringently washed to remove PCR inhibitors and all other undesired solutes. Finally, the captured miRNAs are eluted from the beads, circularized and quantified using SomaGenics' miR-ID® RT-qPCR assays. As a result, miR-Direct® provides higher sensitivity than other methods, allowing reliable and reproducible quantification of miRNAs with very low abundance. In addition, miR-ID® is uniquely capable of discriminating miRNA isoforms and isomiRs that vary in sequence or length by as little as one nucleotide along any position of the miRNA sequence, outperforming other qPCR methods in this respect. Sample processing up to the reverse transcription step is performed in a single tube. miRNA levels can be measured regardless of whether the samples were collected in EDTA, citrate, or heparin, whereas most commercially available purification methods are incompatible with heparin-containing plasma. Here we present data showing the application of miR-Direct® for quantification of miRNAs in plasma, serum, whole blood, and urine. miR-Direct® may be used for validating miRNA sequencing data and biomarker candidates as well as for developing diagnostic (e.g. liquid biopsy) and prognostic applications for circulating miRNAs, including canonical and non-canonical miRNA forms such as isomiRs.

miRNA as biomarkers



modified from: Sundarbose et al., Liver Transplantation, 2012

Current problems with miRNA isolation from biofluids

Column	Organic Extraction	Ethanol precipitation
• Incomplete removal of RT and PCR inhibitors • Incomplete and inconsistent adsorption to and release from columns • Sample recovery varies with GC content	• Incomplete removal of RT and PCR inhibitors • Incomplete phase separation • Selective sample loss of miRNAs with low GC content and/or secondary structure	• Incomplete removal of RT and PCR inhibitors • Sample loss from low concentration of miRNAs and incomplete RNA precipitation

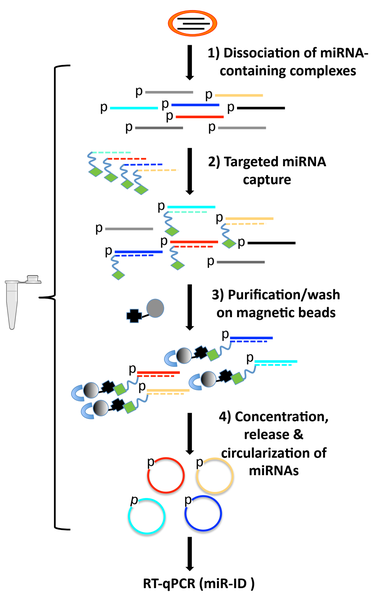
McAlexander et al, 2013, Frontiers in Genetics
Burgos et al, 2013, RNA
Kim, Y.K. et al, 2010 Mol Cell

Challenges in quantifying cf-miRNAs

1. Pre-analytical variables
   - Sample collection
   - Avoid/screen for hemolysis
   - Sample storage prior to analysis
2. Reproducible recovery of miRNAs from biofluids
   - Quantitative/efficient release of miRNAs from protective complexes
   - Once released, miRNAs must be protected from abundant ribonucleases
   - Deficiencies with standard methods of RNA extraction
3. Sensitivity and specificity of qPCR detection methods
   - Reliable measurement of low to moderately abundant miRNAs
   - Accurate quantification of miRNA isoforms, isomiRs, and novel miRNAs
4. Data normalization and analysis
   - Not standardized
   - No universal "invariant" endogenous miRNA control
   - Spike-in miRNAs may be used for technical replicate quality control

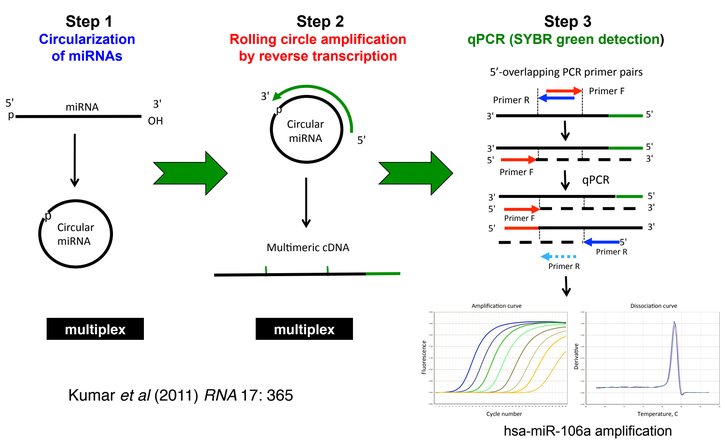
miR-Direct®/miR-ID®

miR-Direct® multiplex workflow diagram



- ○ miRNA capture from variable input volumes (25 to 400 µl)
- ○ No solvent extraction or column purification
- ○ Front-end processing is performed in a single tube
- ○ Efficiently removes inhibitors of enzymatic reactions (including heparin)
- ○ miRNA concentration without total RNA purification
- ○ Steps until qPCR can be multiplexed for all miRNAs of interest

miR-ID®

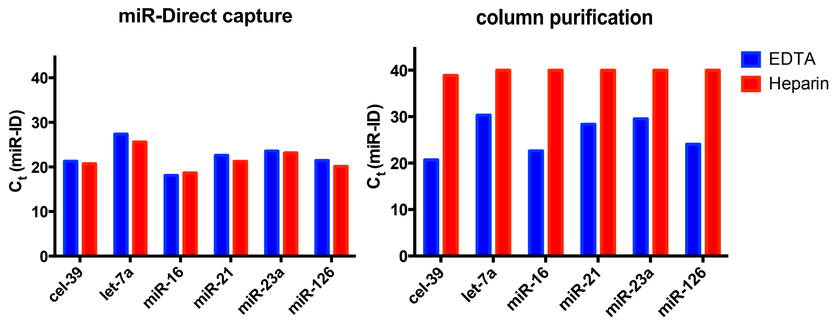


Kumar *et al* (2011) *RNA* 17: 365

hsa-miR-106a amplification

- Highly sensitive qPCR method (8 log dynamic range)
- Multiplex circularization and cDNA synthesis prior to qPCR
- Superior SNP discrimination at any position on the miRNA
- Discrimination of 5'- and 3'- isomiRs
- Distinguishes 3'-end modifications (2'-OMe/2'-OH)

miR-Direct® eliminates inhibitors of enzymatic reactions

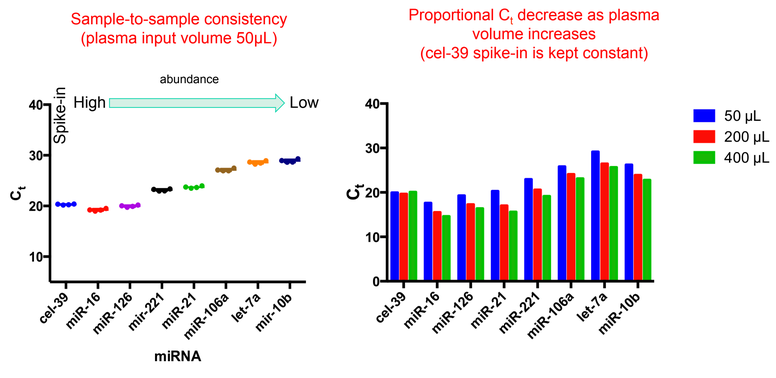


miR-Direct® is not inhibited by heparin

- C_t levels of miRNAs that were **not detected** were set to 40 (max. number of cycles run)

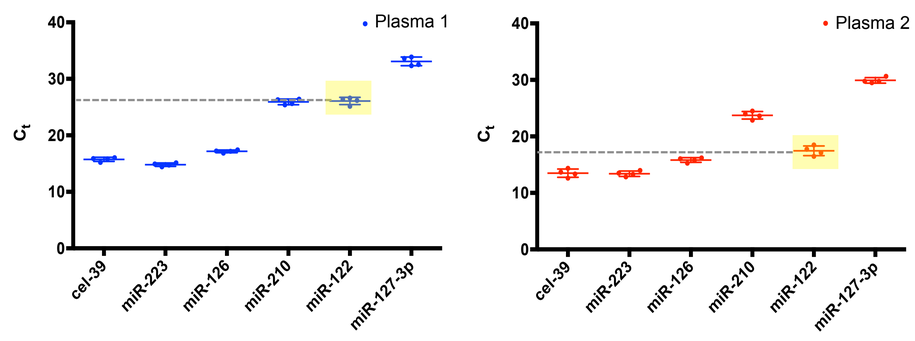
Donor matched plasma samples collected with EDTA or heparin were purified by miR-Direct (left panel) or column kit (right panel). miRNAs were analyzed using miR-ID® RT-qPCR. 200 µL biofluid was input into miR-Direct assay, 8 µL plasma equivalent was input into the column-purified samples.

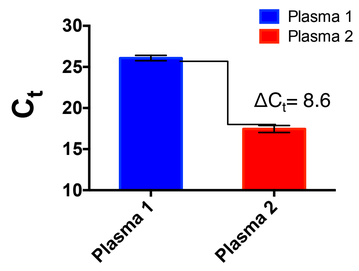
miR-Direct® detection of miRNAs in plasma is quantitative and proportional



- Allows high input plasma volume with no enrichment of blood-borne enzyme inhibitors
- Spike-in miRNA controls can be included to control for sample-to-sample variability

Detection of different levels of miR-122 miRNA in 2 plasma donors using miR-Direct®

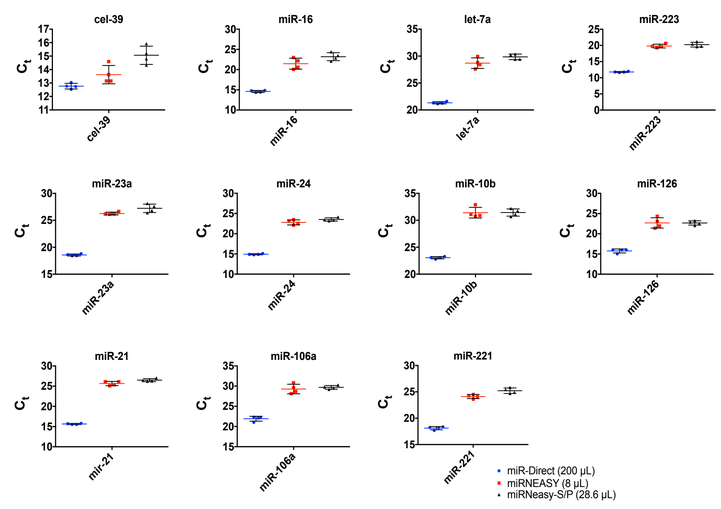




miR-Direct (200 µL plasma)

miR-122

miR-Direct® has less inter-assay variability and greater sensitivity than column-purification methods

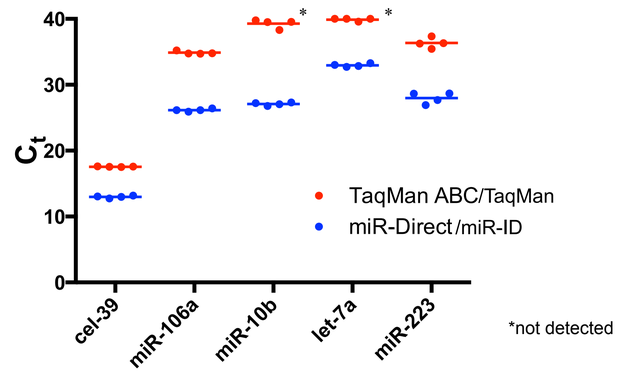


Increased sample input for column purification does not result in increased sensitivity

	Predicted ΔCt	Actual ΔCt (range)	ΔΔCt (range)
miR-Direct (200 µL) vs miRNeasy (8 µL)	4.6	6-8.3	1.4-3.7
miR-Direct (200 µL) vs miRNeasy S/P (28.6 µL)	2.8	7-10.9	4.2-8.1

Technical reproducibility and sensitivity is examined by miR-Direct (blue), miRNeasy column purification (red), and miRNeasy Serum/Plasma kit column (black) of 4 biological replicates of plasma collected from the same donor. miR-Direct was performed using 200 µL plasma input determined by analyzing four 200-µl aliquots of the same plasma whereas the equivalent of 8 µL plasma was input into the miRNeasy-purified samples versus 28.6 µL . miR-ID RT-qPCR is used for all purification methods so that the data can be directly compared. The difference between the predicted and actual ΔCts reflects that recovery and detection is more quantitative by miR-Direct.

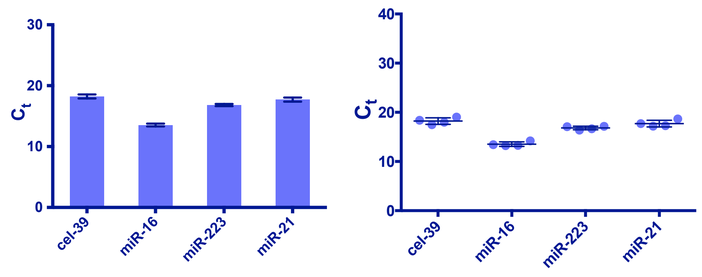
miR-Direct® can be used for profiling miRNAs in urine



*not detected

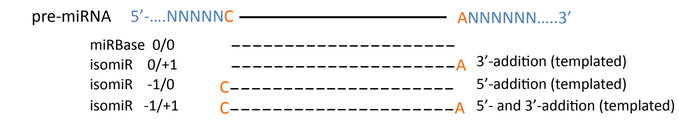
- Analysis of urine samples collected from 4 healthy volunteers
- Comparison of miR-Direct with TaqMan ABC kit
- C_t levels of miRNAs that were not detected were set to 40 (max. number of cycles run)

miR-Direct® robustly detects miRNAs in whole blood



4 replicates starting with 50 µL whole blood from a single donor

miR-ID® discrimination of an isomiR family derived from a common pre-miRNA

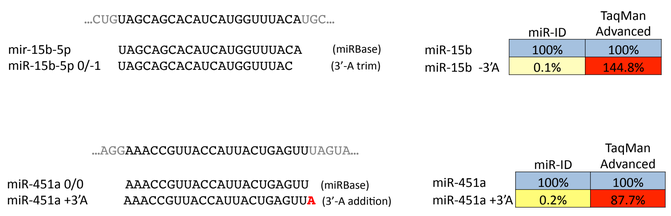


	miR-ID® assays			
	0/0	0/+1	-1/0	-1/+1
0/0 (mature)	100%	0.09%	1.72%	0.00%
0/+1 isomiR	0.09%	100%	0.20%	0.17%
-1/0 isomiR	0.75%	0.72%	100%	0.00%
-1/+1 isomiR	0.01%	0.05%	0.13%	100%

	TaqMan Advanced
	0/0
0/0 (mature)	100%
0/+1 isomiR	37.89%
-1/0 isomiR	8.89%
-1/+1 isomiR	21.53%

Cross-discrimination assays using a synthetic reference miRNA and its isomiRs by miR-ID® (top) and TaqMan Advanced Assays (bottom). -1, 0, +1 symbolize upstream, no change, or downstream terminus shifts with respect to the parent miRNA at the 5' or 3' ends. TaqMan Advanced assays include a poly-A addition step to the 3'-end of the miRNA, resulting in reduced discrimination power of 3'-A additions to miRNAs.

Discrimination of 3'-addition and 3'-deletion isomiRs



	miR-ID	TaqMan Advanced
miR-15b	100%	100%
miR-15b -3'A	0.1%	144.8%

	miR-ID	TaqMan Advanced
miR-451a	100%	100%
miR-451a +3'A	0.2%	87.7%

- Adenine additions are the most prevalent sequence modifications found in isomiRs
- qPCR methods with a polyA-tailing step (e.g. TaqMan Advanced) cannot discriminate adenine polymorphisms at the 3'-end of a miRNA

Summary

- ❖ Convenient workflow
  - miRNA release and capture takes place in a single tube
  - No liquid/liquid extraction or column purification needed
  - Amenable to automation
- ❖ Superior sensitivity
  - Can assay large input sample volumes
  - Expands the number of miRNAs that could be part of a biomarker signature
- ❖ Superior reproducibility
  - Minimal number of volume transfers decreases intra- and inter-assay variability
  - Eliminates variable recovery from liquid/liquid extraction or column purification
- ❖ Superior specificity
  - Incorporation of miR-ID® detection allows for SNP discrimination and isomiR-specific quantification

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