miR-Direct™: Quantification of Circulating miRNAs Directly from Plasma or Serum

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Abstract

Circulating microRNAs (miRNAs) have great potential as biomarkers, but current methods for their quantification are hampered by inconsistent RNA recovery during isolation, by the difficulty of eliminating RT-PCR inhibitors (including heparin), and by the low concentration at which most miRNAs appear in blood. We have developed a novel method (miR-Direct™) in which miRNAs of interest can be recovered from plasma or serum samples and quantified by RT-qPCR without prior purification of total RNA. This assay can be used for discovery and validation of circulating miRNA biomarkers, especially in preclinical and clinical studies. First, samples (25-400 µl) are treated to release miRNAs from complexes with lipids or proteins. Next, miRNAs of interest are captured using miRNA-specific probes and magnetic beads. After washing steps, the miRNAs are eluted from the beads in a minimal volume and quantified by RT-qPCR, using SomaGenics' circularization-based miR-ID® assays. There are no organic extraction or ethanol precipitation steps, thus avoiding procedures that lead to inconsistent recovery of small RNAs. Because miR-Direct simultaneously concentrates the miRNAs in biofluids and washes away potential PCR inhibitors, more starting material can be input into each assay, resulting in greater sensitivity and accuracy than is seen with standard methods. miRNA levels can be measured regardless of whether the samples were collected in EDTA, citrate, or heparin, whereas most assays are incompatible with heparin-containing plasma. Sample processing up to the reverse transcription step is performed in a single tube, so the method is highly amenable to automation. In addition to miRNAs found in circulation, the miR-Direct approach can potentially be adapted to assay any small RNAs (or fragments of large RNAs) present in any biological fluid. It can also be used to quantify endogenous small RNAs from cell or tissue lysates.

Circulating miRNAs are protected from nuclease degradation

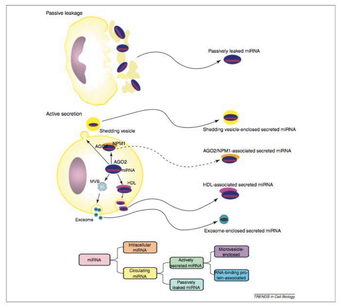


Figure adapted from Chen et al, (2012) Trends in Cell Biology 22:125

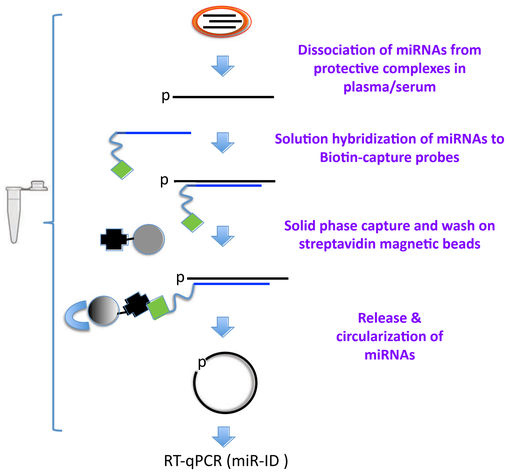
- Encapsulation in microvesicles/exosomes
- Binding to proteins (Ago2)
- Binding to lipoprotein particles (HDL & LDL)

Challenges in quantifying circulating miRNAs

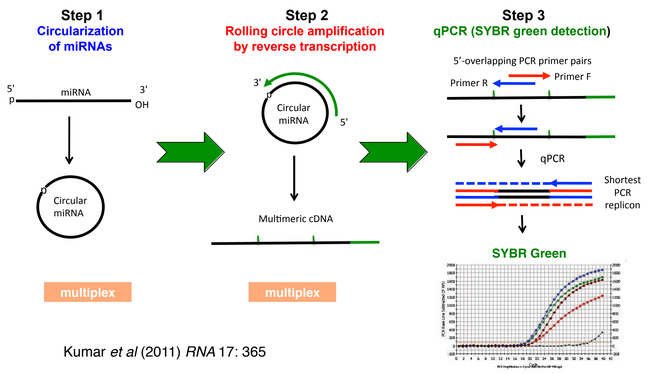
- Release miRNAs from complexes while protecting them from abundant ribonucleases
- Spike-in controls must also be protected from nucleases
- Recovery of miRNAs from biofluids by conventional total RNA isolation methods is incomplete and variable (<50%)*
- Reliable measurement of low to moderate abundance of miRNAs
  - May require large input volumes of plasma/serum
  - Concentrating the the sample also concentrates PCR inhibitors present in blood
- Poor correlation of miRNA levels across platforms (qPCR, microarray, NGS)
- Important to minimize variability during
  - Blood draw and storage
  - Sample processing

*Burgos *et al* (2013) RNA 19:712

miR-Direct scheme

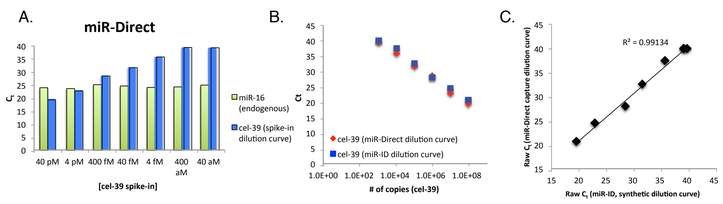


miR-ID®



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

miRNAs are quantitatively captured from plasma

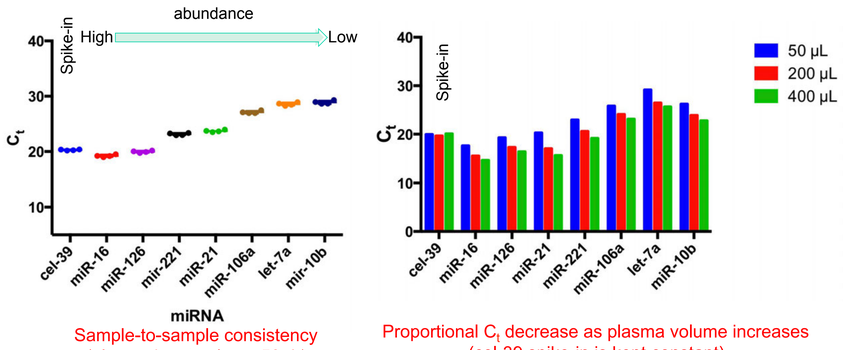


A. Spike-in dilution curve (cel-39) in 400 µL plasma samples are analyzed by miR-Direct. C_t values for endogenous miR-16 are constant across samples

B. To assess efficiency of miR-Direct miRNA-probe hybridization, cel-39 was serially diluted and spiked into 400 µL plasma for analysis by miR-Direct (red) or it was diluted into H_2O for miR-ID analysis (blue) without addition of a capture probe.

C. Pair-wise analysis of raw C_ts obtained with and without capture of cel-39 spike-in (data from panel B.

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

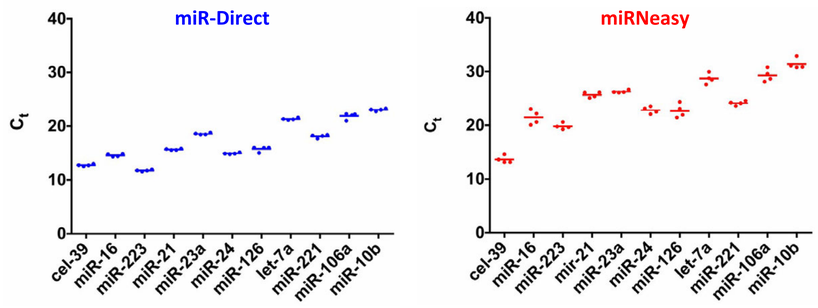


Sample-to-sample consistency (plasma input volume 50µL)

Proportional C_t decrease as plasma volume increases (cel-39 spike-in is kept constant)

- Allows high input plasma volume with no enrichment of blood-borne enzyme inhibitors
- Measured miRNA levels increase proportionately to the input plasma volume
  - ~2C_t decrease in every 4-fold increase in plasma volume
- Spike-in miRNA controls can be included to control for sample-to-sample variability

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

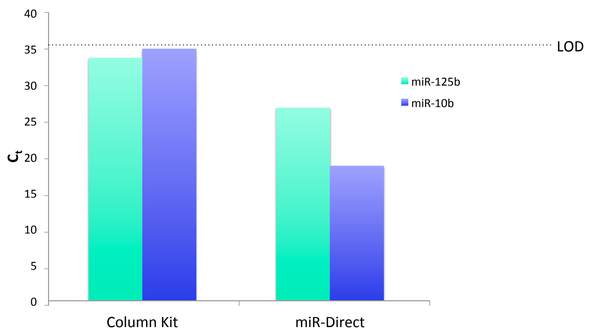


Comparison of CV (expressed as %) for miR-Direct vs. column purification

	cel-39	miR-16	miR-223	miR-21	miR-23a	miR-24	miR-126	let-7a	miR-221	miR-106a	miR-10b
miR-Direct	1.65%	1.74%	1.38%	0.85%	0.99%	0.94%	3.10%	0.92%	1.67%	2.75%	0.88%
miRNeasy	4.99%	6.33%	2.90%	2.07%	0.97%	2.76%	5.65%	3.43%	1.63%	4.04%	3.16%

Sample-to sample reproducibility is examined by miR-Direct (left) and miRNeasy column purification (right) 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 column-purified RNAs. miR-ID qPCR was used in both panels. miR-Direct shows a ~7-10 C_t improvement over miRNeasy-purified samples. The 50-fold larger plasma volumes used by miR-Direct should contribute a theoretical ~5.5 C_t advantage. The remainder may reflect more efficient capture of miRNAs by miR-Direct over column methods.

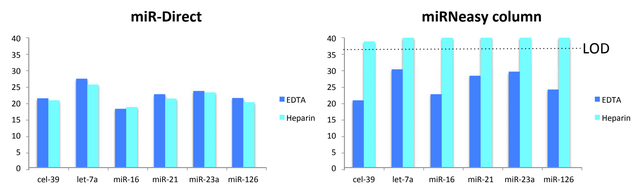
Increased plasma input allows for detection of low abundance miRNAs in plasma that are not quantifiable by conventional column purification



miRNeasy column-based kit purification using 8µl equivalent plasma volume with TaqMan detection is compared with miR-Direct assay starting with 200 µL plasma volume.

LOD= limit of detection

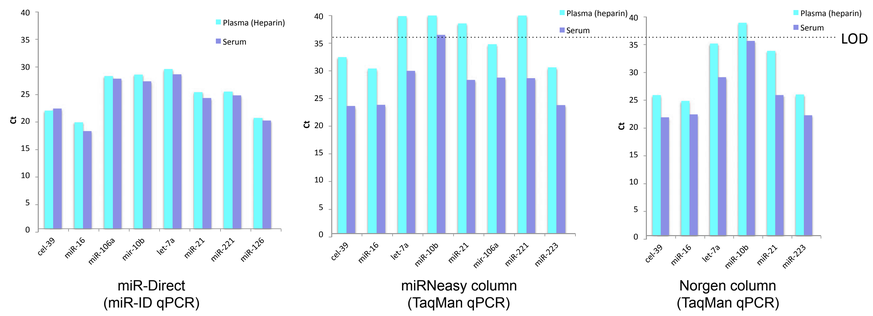
miR-Direct can detect miRNAs even in plasma samples stabilized by heparin



- Heparin is a widely-used anticoagulant for blood plasma collection
- However, it is a known inhibitor of enzymatic reactions associated with qPCR
  - co-purified by the most often used column-based purification kits because it is a highly negatively charged molecule
- Major kits on the market recommend against the use of plasma collected with heparin for further analysis (qPCR, Nanostring, NGS)

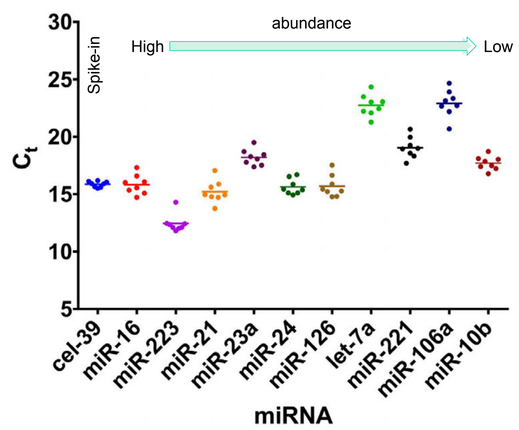
- Plasma was obtained from same donor using either EDTA- or heparin-containing collection tubes
- miR-Direct allows for direct miRNA quantification in heparin-containing plasma
- Conventional assay using isolated total RNA from columns fails in presence of heparin

miR-Direct provides comparable results for plasma and serum samples



Plasma (heparin) and serum were collected from same donor at the same time and analyzed by the indicated methods. 200 µL biofluid was input into miR-Direct assay, 8µL biofluid equivalent was input into the column-purified samples.

miR-Direct on plasma from 8 different donors: natural variation



miR-Direct assays on plasma collected from 8 different donors shows natural variability in miRNA levels from person to person.

Summary

Advantages of miR-Direct™ in quantifying circulating miRNAs:

- ✓ **Can quantify low-abundance miRNAs in circulation by assaying large input sample volumes**
  - Increases overall sensitivity
  - 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
- ✓ **Process can be automatable**
  - miRNA release and capture takes place in a single tube
  - No liquid/liquid extraction or column purification needed
- ✓ **Eliminates potential blood-borne enzyme inhibitors as well as heparin**

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