



# Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR)

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## ARTICLE INFO

### Article history:

Accepted 26 January 2010

Available online 8 February 2010

### Keywords:

microRNA  
Biomarker  
Circulating  
qRT-PCR  
miRNA

## ABSTRACT

MicroRNAs (miRNAs) are small (~22 nt) RNAs that play important roles in gene regulatory networks by binding to and repressing the activity of specific target mRNAs. Recent studies have indicated that miRNAs circulate in a stable, cell-free form in the bloodstream and that the abundance of specific miRNAs in plasma or serum can serve as biomarkers of cancer and other diseases. Measurement of circulating miRNAs as biomarkers is associated with some special challenges, including those related to pre-analytic variation and data normalization. We describe here our procedure for qRT-PCR analysis of circulating miRNAs as biomarkers, and discuss relevant issues of sample preparation, experimental design and data analysis.

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## 1. Introduction

MicroRNAs (miRNAs) are small, non-protein-encoding RNAs that post-transcriptionally regulate gene expression via suppression of specific target mRNAs [1,2]. Recently, we and others have demonstrated that miRNAs circulate in a highly stable, cell-free form in the blood (i.e., they can be detected in plasma and serum) [3–9]. Furthermore, tumor cells have been shown to release miRNAs into the circulation [3] and profiles of miRNAs in plasma and serum have been found to be altered in cancer and other disease states [3,4,7,8], suggesting broad opportunities for development of circulating miRNAs as blood-based markers for molecular diagnostics.

Prerequisite to developing circulating miRNA-based diagnostics is the ability to measure miRNAs from plasma and/or serum with sufficient sensitivity and precision to be clinically effective. The small size of the mature miRNA sequence (~22 nt) and sequence homology between the mature and precursor miRNA forms has required advances in PCR-based detection methods for the quantitative analysis of miRNAs from cultured cells and tissue specimens. These challenges have been met by innovative solutions based on qRT-PCR, as published elsewhere [10–12].

Adapting miRNA qRT-PCR technology to the analysis of circulating miRNAs, however, requires modified RNA extraction methods to

permit use of human plasma or serum as the starting material, dealing with the difficulties in quantifying the minute amounts of RNA typically recovered from plasma or serum, and data normalization to correct for technical variations in the entire procedure. We focus here on discussing these issues, as well as describing a protocol that we have used for circulating miRNA qRT-PCR that is based on commercially available TaqMan technology.

## 2. Description of methods

### 2.1. Overview

The clinical effectiveness of circulating miRNAs as biomarkers is likely to be affected by a range of variables including pre-analytic factors having to do with specimen collection and processing, factors influencing RNA extraction efficiency, and the technical issues involved in successful qRT-PCR and data analysis. We will begin with a discussion of issues to be considered at the various steps of this pipeline, followed by a presentation of the detailed protocol that we have used successfully in our laboratory for carrying out extraction and qRT-PCR of miRNAs from clinical plasma and serum specimens.

### 2.2. Pre-analytic considerations

#### 2.2.1. Plasma and serum collection

Plasma is the cell-free supernatant obtained after centrifuging blood that has been collected in the presence of an anticoagulant. Serum is the cell-free supernatant obtained after centrifuging

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blood that has been allowed to spontaneously clot, after being collected in the absence of anticoagulant. The protocols for plasma and serum preparation, though in theory standard processes in clinical labs, are subject to variability at several steps. For example, when generating plasma there is a risk of contamination of the plasma supernatant by cells from the cellular pellet when aspirating; as cells have a much higher concentration of miRNA than plasma or serum, cellular carryover has the potential to be a significant confounding factor if care is not taken during blood processing. The type of anticoagulant used in plasma collection tubes is also important to consider. Whereas EDTA and citrate are both acceptable anticoagulants for downstream qRT-PCR, the use of heparin as the anticoagulant potentially inhibits subsequent PCR (data not shown).

With regard to the collection of blood for generating serum, at least two alternative types of tubes are in common use: a simple tube in which the clotted blood is simply centrifuged with recovery of supernatant (serum), and alternatively a serum separator tube in which a gel is included that allows more discrete separation of the clot from serum during the centrifugation process. Although both are effective, it is important that the type of collection tube used be standardized within a study to avoid confounding the results.

### 2.2.2. Other pre-analytic variables

There are other pre-analytic variables that have yet to be studied carefully. Although miRNAs appear to be stable to extended room temperature incubation of plasma (at least up to 24 h) [3,6] it is not yet known whether the duration of time taken between blood collection and processing of plasma or serum affects miRNA levels. In our studies, we have typically worked with specimens that have been processed to generate plasma or serum within 4 h of collection. In the absence of data on this pre-analytic variable, it is prudent in designing studies to standardize conditions as much as possible with respect to time elapsed between whole blood collection and processing for plasma or serum.

Additional variables for future study include: diurnal variation in miRNA levels, fasting vs. non-fasting state at blood collection (fatty meals can cause lipemia in the blood that could affect RNA extraction efficiency), white blood cell count, gauge of needle used for phlebotomy, and whether the skin plug obtained in the blood tube collected at the initial skin puncture contributes a significant amount of confounding miRNA. These are factors that may plausibly affect either the amount of miRNA present in a given plasma or serum sample, the ability to extract RNA efficiently, or the ability to measure it robustly by qRT-PCR (i.e., if inhibitors are present). Some of these factors (such as RNA extraction efficiency and effect of PCR inhibitors) can be controlled for by the use of synthetic, non-human (e.g., *Caenorhabditis elegans*) miRNAs spiked in as controls at the onset of RNA isolation (described in Section 2.5); however, in the absence of data on the influence of such variables, it is prudent to try to match as many variables as possible in the collection and/or selection of case and control samples for research studies.

Storage of plasma or serum at  $-80^{\circ}\text{C}$  appears to be acceptable and freeze-thawing does not appear to be a significant factor, at least for the abundant miRNAs examined [3,6]. Although we routinely analyze specimens that are several years old, the effect of duration of storage on miRNA levels has not yet been carefully evaluated; thus it is best to match case and control specimens with respect to duration of storage as much as possible.

### 2.2.3. Plasma vs. serum

Both plasma and serum are acceptable types of specimen for circulating miRNA analysis. In a pilot experiment using three miRNAs, we found miRNA measurements between plasma and serum to be highly correlated [3]. However, mixing specimen types with

in a study is not recommended, given that a global comparison of miRNA expression between plasma and serum has not yet been performed.

### 2.3. RNA extraction considerations for plasma and serum specimens

Plasma and serum are biospecimens that have a very high concentration of protein. Extraction therefore currently involves scaling up the volume by diluting with one or more volumes of denaturing solutions. We have used variations on two commercially available kits: Ambion mirVana PARIS (in which the only modification is that we add an additional organic extraction step), and the Qiagen miRNeasy kit, in which the modification is simply using 10 volumes of Qiazol reagent to initially denature 1 volume of plasma or serum. Although both protocols have proven effective, in our hands the Qiagen protocol appears to produce 2–3-fold greater RNA yield (data not shown). Other approaches such as one using the Trizol LS reagent have also been reported to be effective [4,5]. A major unmet challenge with the current protocols is difficulty in scaling the procedure up to larger plasma volumes, given that dilution with significant volumes of denaturing reagent is required for these protein-rich specimens.

There is considerable sample-to-sample variability in both protein and lipid content of plasma and serum samples, which could affect efficiency of RNA extraction, and could introduce potential inhibitors of PCR. In order to adjust for such variations in RNA extraction and/or in co-purification of inhibitors, we routinely spike-in non-human (e.g., *C. elegans*) synthetic miRNAs after the initial denaturation of plasma or serum (see protocol below). These go through the entire RNA isolation process and are ultimately measured by qRT-PCR in the final RNA eluate, providing an internal reference for normalization of technical variations between samples.

Furthermore, the yield of RNA from small volume plasma or serum samples (i.e., 100–400  $\mu\text{L}$ ) has, in our hands, been below the limit of accurate quantitation by spectrophotometry. The inclusion of the spiked-in oligos is therefore an important part of our protocol for adjusting for differences in efficiency of RNA recovery between samples, as we use a fixed volume of eluted RNA sample as input for qRT-PCR, rather than using a fixed mass of input RNA.

### 2.4. RNA quality control and normalization controls

As described in Section 2.3, it is possible to normalize for some of the *technical* variability of the plasma or serum RNA extraction using the *C. elegans* spiked-in control miRNAs. An effective normalization strategy for *biological* variability, however, is currently not well-developed because the factors that lead to biological variation independent of the disease being studied are not known, and their effect on specific miRNAs has not been characterized. At present, we cannot endorse any specific miRNA or set of miRNAs as suitable endogenous controls. The use of so-called “invariant” miRNAs as endogenous controls has been proposed by some investigators. Although this may not be detrimental, as invariant miRNAs as normalizers tend not to affect the data significantly, at the same time this may not correct for true biological variability which may be reflected in specific miRNAs that may not be the most invariant. That said, the use of typically invariant miRNAs can be useful as a quality control check to identify specimens that may be of low quality relative to others. For this purpose we have used miR-16 and miR-223 in our laboratory, as both are expressed at high levels in plasma and serum and are relatively invariant across large numbers of samples. In future work, we anticipate improved sets of such miRNAs may be assembled for quality control of RNA isolated from plasma or serum.

### 2.5. Protocol: isolation of total RNA from plasma or serum

We have adapted the protocol from the miRNeasy RNA isolation kit (Qiagen, Inc.) as follows to permit isolation of total RNA from plasma and serum specimens:

1. Plasma and serum samples are thawed completely on ice. Each sample is always mixed by inversion immediately prior to aliquoting for RNA isolation, in order to evenly disperse any particulates that may be present. Vortexing prior to denaturation should be avoided. A volume of 400  $\mu\text{L}$  serum or plasma permits convenient handling throughout the procedure and has been sufficient to detect cancer-derived circulating miRNA biomarkers in advanced prostate cancer sera. Smaller volumes (e.g., 25  $\mu\text{L}$ ) can be used if highly abundant circulating miRNAs are being measured.
2. To ensure effective denaturation of proteins, add 10 volumes of Qiazol solution to one volume of plasma or serum, mix well by vortexing, then incubate at room temperature for 5 min.
3. Add 5  $\mu\text{L}$  of a pool of three synthetic *C. elegans* miRNAs from a 5 fmol/ $\mu\text{L}$  stock tube. Vortex each sample immediately after addition of the miRNAs.<sup>2</sup>
4. Aqueous and organic phase separation is achieved by addition of 0.2 volumes molecular grade chloroform. To obtain a stable, clear aqueous phase, it is critical to thoroughly homogenize the denatured sample by vortexing at the maximum setting for  $\geq 30$  s, followed by centrifugation at 12,000g for 15 min at 4 °C. Working quickly, transfer the aqueous phase to a new tube, carefully avoiding the interphase altogether.
5. Continue with the manufacturer's protocol as described. Total RNA is eluted from the column by two sequential elutions with 52.5  $\mu\text{L}$  water to yield  $\sim 100$   $\mu\text{L}$  RNA eluate.

### 2.6. Protocol: single-plex qRT-PCR of miRNAs from plasma or serum RNA

We describe here a protocol for TaqMan-based qRT-PCR that we have adapted for the measurement of circulating miRNAs in our laboratory, using scaled-down (i.e., 5  $\mu\text{L}$ ) reaction volumes. Other protocols for miRNA qRT-PCR are in existence [11,12] and could be adapted for circulating miRNA analysis as well.

Design of the experiments should include avoidance of bias related to batch effects and geographic location of samples in the thermocycler block. Prior to RNA isolation, all samples within a study set should be carefully ordered to alternate sample types that will be compared (e.g., case and control samples) in order to avoid such biases. Sample alternation should be maintained throughout RNA extraction and qRT-PCR steps.

#### 2.6.1. Reverse transcription

Reverse transcription reactions are performed using the TaqMan miRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Part No. 4366597, Applied Biosystems, Inc.) in a scaled down (5  $\mu\text{L}$ ) RT reaction:

1. Each reaction should be comprised of 1.387  $\mu\text{L}$   $\text{H}_2\text{O}$ , 0.5  $\mu\text{L}$  10 $\times$  Reverse-Transcription Buffer, 0.063  $\mu\text{L}$  RNase-Inhibitor (20 U/ $\mu\text{L}$ ), 0.05  $\mu\text{L}$  100 mM dNTPs with dTTP, 0.33  $\mu\text{L}$  Multiscribe

<sup>2</sup> Note: Synthetic miRNAs are added specifically after denaturation of the sample, in order to avoid degradation by endogenous plasma/serum RNases. *C. elegans* synthetic miRNA working stocks, stored at  $-80$  °C, should be thawed on ice and mixed prior to use. Aliquots should be prepared for single use only to avoid variability introduced by multiple freeze/thaw cycles. *C. elegans* synthetic miRNA sequences for use as spiked-in controls: Cel-miR-39, 5'-UCACCGGGUG UAAAUACGCUUG-3'; Cel-miR-54, 5'-UACCCGUAUAUUAUCCAG-3'; Cel-miR-238, 5'-UUUGUACUCCGAUGCCAUUCAGA-3'.

Reverse Transcriptase, 1  $\mu\text{L}$  RT primer. These components should be prepared as a larger master mix. Mix by inversion (do not vortex), and collect contents by brief centrifugation.

2. Aliquot master mix into 0.2 mL RNase-free striptubes or a 96-well plate and add 1.67  $\mu\text{L}$  input RNA. For generation of standard curves using chemically synthesized RNA oligonucleotides corresponding to known miRNAs, serially dilute the synthetic miRNAs as described in Section 2.8 and add to RT reactions at a volume of 1.67  $\mu\text{L}$  per reaction.
3. Mix RT reactions by inversion and centrifuge to collect contents. Use a Tetrad2 Peltier Thermal Cycler (BioRad) to carry out the RT reactions using the following conditions: 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, hold at 4 °C. RT products can be stored undiluted at  $-20$  °C prior to running the real-time PCR.

#### 2.6.2. Real-time PCR

Real-time PCR reactions are performed in duplicate, in scaled-down (5  $\mu\text{L}$ ) reaction volumes using 2.5  $\mu\text{L}$  TaqMan 2 $\times$  Universal PCR Master Mix with No AmpErase UNG, 0.25  $\mu\text{L}$  miRNA-specific primer/probe mix, and 2.25  $\mu\text{L}$  diluted RT product per reaction.

1. For each miRNA-specific assay, prepare a reaction pre-mix by combining sufficient TaqMan 2 $\times$  Universal PCR Master Mix and primer/probe mix for all reactions, plus excess for losses associated with pipetting. Mix by inversion and centrifuge briefly.
2. Aliquot enough reaction mix for two duplicate reactions per sample into striptubes (i.e., 5.5  $\mu\text{L}$  reaction mix plus 15% excess for pipetting losses, per aliquot, will be sufficient for duplicate reactions for a sample, because 2.75  $\mu\text{L}$  reaction pre-mix is needed per final reaction).
3. Dilute the RT products by combining 5.0  $\mu\text{L}$  RT product with 28.9  $\mu\text{L}$  water (1:15 final dilution in PCR reaction). Mix and centrifuge briefly.
4. For each sample, add the diluted RT product to the reaction pre-mix aliquots from step 2, adding enough for duplicate reactions (i.e., 4.5  $\mu\text{L}$  diluted RT product plus 15% excess, because 2.25  $\mu\text{L}$  diluted RT product is needed per PCR reaction). Mix the duplicate reactions, centrifuge, and aliquot 5  $\mu\text{L}$  reactions in duplicate into the optical plate. Seal with ABI MicroAmp™ Optical Adhesive Film. Centrifuge plate to ensure no bubbles inhibit signal detection.
5. Real-time PCR is carried out on an Applied Biosystems 7900HT thermocycler (Applied Biosystems, Inc.) using the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by a hold at 4 °C. Raw data can then be analyzed with SDS Relative Quantification Software version 2.2.3 (Applied Biosystems, Inc.), generally using the automatic cycle threshold (Ct) setting for assigning baseline and threshold for Ct determination.

#### 2.7. Pre-amplification of RT products (optional)

If desired, the RT product can be pre-amplified prior to the real-time PCR step to potentially enhance sensitivity. The pre-amplification step is carried out in a small-scale (5  $\mu\text{L}$ ) reaction comprised of 2.5  $\mu\text{L}$  Taqman PreAmp Master Mix (2 $\times$ ), 1.25  $\mu\text{L}$  Taqman miRNA assay (0.2 $\times$ ) (diluted in TE) and 1.25  $\mu\text{L}$  undiluted RT product from Section 2.6.1.

1. For each miRNA-specific assay, prepare a reaction pre-mix by combining sufficient Taqman PreAmp Master Mix (2 $\times$ ) and miRNA assay (0.2 $\times$ ) for all reactions, plus excess for losses associated with pipetting. Mix by inversion and centrifuge briefly.
2. Aliquot 3.75  $\mu\text{L}$  pre-mix into 0.2 mL RNase-free striptubes.

- For each sample, add 1.25  $\mu\text{L}$  of the undiluted RT product to the reaction pre-mix aliquots from step 2. Mix by inversion and centrifuge briefly.
- Preamplification is carried out on a Tetrad2 Peltier Thermal Cycler (BioRad). Cycling conditions: 95  $^{\circ}\text{C}$  for 10 min, followed by 14 cycles of 95  $^{\circ}\text{C}$  for 15 s and 60  $^{\circ}\text{C}$  for 4 min, followed by a hold at 4  $^{\circ}\text{C}$ .
- Dilute the pre-amplification PCR product by adding 20  $\mu\text{L}$  water to the 5  $\mu\text{L}$  pre-amplification reaction product. Mix by inversion and centrifuge briefly. Proceed with the real-time PCR protocol as described for non-pre-amplified samples in Section 2.6.2 above, using the dilute pre-amplified product as the reaction input, at 2.25  $\mu\text{L}$  per reaction.<sup>3</sup>

### 2.8. Absolute quantification of miRNAs using synthetic miRNA standards

The generation of a standard curve (using synthetic miRNA target), carried out in parallel with qRT-PCR of biological samples, provides a means to estimate absolute copy number of the target miRNA in biological samples. This is with the caveat that synthetic oligonucleotides diluted in water may perform differently than endogenous miRNAs isolated from plasma or serum RNA, given that in the latter case a background of other RNAs is expected to be present in the sample.

- Starting with a 1 nM solution of synthetic HPLC-purified, 5' phosphorylated oligonucleotide (Integrated DNA Technologies, Inc.) with identical sequence to the mature miRNA of interest, a primary dilution of 8.192 fM is created by adding 4  $\mu\text{L}$  of the 1 nM solution to 118.1  $\mu\text{L}$  H<sub>2</sub>O. This solution is mixed and centrifuged.
- From that initial dilution, 10 serial 4-fold dilutions are made by adding 20  $\mu\text{L}$  of the previous solution to 60  $\mu\text{L}$  RNase-free, DNase-free H<sub>2</sub>O, such that the 11th tube contains ~10 copies of miRNA mimetic. The 12th tube should contain H<sub>2</sub>O only and is used as a no-template control.
- qRT-PCR of synthetic miRNA serial dilutions should be run in parallel (i.e., on the same PCR machine block) with experimental samples, beginning from the reverse transcription step onwards, using the same master mixes and real-time PCR optical plate. It is important to avoid cross-contamination of biological samples by the highly concentrated synthetic oligonucleotide dilutions; it is advisable to separate them by an empty row on the plate.
- Plotting Ct values versus copy number of the synthetic miRNA in a standard curve allows fitting of a curve that is then used to approximate copies of endogenous miRNA from Ct values obtained with biological samples.

### 2.9. Normalization of experimental qRT-PCR data using spiked-in synthetic *C. elegans* miRNAs as controls

We normalize the data across samples using a median normalization procedure:

- Run TaqMan qRT-PCR assays in duplicate for each of the three synthetic spiked-in *C. elegans* miRNAs across all samples in an experimental set. Calculate a mean Ct for each of *C. elegans* synthetic miRNAs for each sample.

- Calculate the median of all the mean *C. elegans* synthetic miRNA Cts, considering all the samples.
- For each sample, calculate a normalization factor by subtracting the mean *C. elegans* synthetic miRNA Ct of the sample of interest from the median value obtained in Step 2.
- Add the normalization factor to the raw Ct value obtained for each assay. The data can now be expressed as median-normalized Ct values.

### 3. Concluding remarks

Here we have described one protocol for extraction and quantification of miRNA from human plasma and serum samples that we have found useful in investigating miRNAs as circulating biomarkers. As detailed above, careful matching of case and control samples and pre-analytic steps is critical given the current paucity of knowledge about the influence of pre-analytic variables on miRNA measurements. In addition, careful experimental design in the layout and performance of qRT-PCR is important for success. Although this article has focused on single-plex qRT-PCR of individual miRNA markers, high-throughput qRT-PCR platforms based on microfluidics and other kinds of liquid handling technology have entered the market and provide an additional tool for circulating miRNA biomarker discovery.

### Acknowledgments

M.T. acknowledges support from a Department of Defense (DOD) Prostate Cancer New Investigator Award (PC074012), a Pacific Ovarian Cancer Research Consortium Specialized Program of Research Excellence (SPORE) in Ovarian Cancer Career Development Award (NIH P50 CA83636; N. Urban PI), a Pilot Award from NIH/NCI Cancer Center Support Grant (5 P30 CA015704), a Pacific Northwest Prostate Cancer SPORE Pilot Grant (NIH P50 CA97186; P. Nelson and J. Stanford co-PIs), a DOD Ovarian Cancer Career Development Award (OC080159), and a Prostate Cancer Foundation Creativity Award. M.T. is a Damon Runyon-Rachleff Innovator supported in part by the Damon Runyon Cancer Research Foundation.

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<sup>3</sup> Note: Pre-amplification products should be used for real-time PCR as soon as possible. Storage for more than a few hours or freezing prior to commencing real-time PCR is not recommended, as we have observed that this compromises sensitivity and precision of the approach.