Reduce microRNA RT-qPCR Assay Costs by More Than 10-fold Without Compromising Results

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Abstract

This white paper describes a detailed protocol for carrying out qPCR-based microRNA analysis for only ~$0.39 per assay, a cost-savings of >90% compared to commonly used alternative methods.

Introduction

Increasingly, NGS is being used as an alternative to microarrays for global small RNA profiling, since NGS offers advantages in terms of the ability to discover novel small RNAs and allows samples from many individuals to be multiplexed for increased throughput and reduced cost. Verification of differentially expressed small RNAs discovered using NGS is usually carried out using a different method, typically reverse transcription quantitative PCR (RT-qPCR). However, the per-assay cost for RT-qPCR can be prohibitive, especially when large panels of candidate microRNAs need to be assessed in many different samples.

Overview of Procedure:

The procedure is based on a method described in the open literature ("Specific and sensitive quantitative RT-PCR of miRNAs with DNA primers"; Balcells et al, BMC Biotechnology 2011, 11:70), which we have optimized and now use routinely at Bioo Scientific for quantitative analysis of microRNAs. The method is sufficiently sensitive to detect microRNAs in cell-free fluids, including urine, saliva, blood plasma and cell culture media. We refer to the method as "miR-specific qPCR".

miR-specific qPCR Protocol:

1. Simultaneous polyadenylation and reverse transcription to make a cDNA library of all microRNAs in the RNA sample, using an RT primer containing a tag sequence at the 5' end.

2. SYBR-based qPCR using Forward primers spanning most of the sequence of the target microRNA and Reverse primers that consist of the RT primer tag sequence at the 5' end, a central poly T region complementary to the added poly A's, and a 3' region consisting of several bases complementary to the 3' end of the target microRNA.

Unlike similar approaches that use simultaneous polyadenylation and reverse transcription to convert microRNA to cDNA, this method does not use a Universal Reverse Primer for the qPCR step; instead it uses microRNA-specific Reverse primers. This important modification provides additional specificity for detection of the intended microRNA targets.

A bottleneck to the method as first described was the time required for design and analysis of the PCR primers. Candidate primer sequences had to be analyzed and optimized for target specificity and Tm compatibility, while minimizing primer-dimer, self-dimer, and

secondary structure. A major breakthrough was the development of a new algorithm for design of the Forward and Reverse PCR primers. The software, which is freely available, enables design of qPCR primers for large panels of microRNAs and other small RNAs, using only the accession numbers as input. See Note 2 below for the link to the software.

Detailed miR-specific qPCR Protocol:

Materials needed are shown in Table 1 below:

Table 1. Materials required for RT-qPCR microRNA detection using miR-specific qPCR method

Reagent	Source	Cost	Number of qPCRs that can be processed with this amount of reagent	Cost per 20 µL qPCR
polyA polymerase (E.coli)	New England Biolabs cat#M0276S (100 units, 5 units/µL)	$63	4,000	$0.016
10 mM ATP	Provided with NEB polyA Pol	$0	>>4,000	NA
10X PolyA buffer	Provided with NEB polyA Pol	$0	>>4,000	NA
4dNTP mix, 10 mM each	NEB cat# N0447S (8 uMol of each, 0.8 mL volume)	$53	240,000	$0.00023
M-MLV Reverse Transcriptase	Bioo Scientific cat#521001 10,000 units at 200 units/µL, ie 50 µL per sales unit	$59	3,000	$0.02
RT primer: 26mer at 10 uM conc 5' CAGGTCCAG TTTTTTTTTTTTTTVN	IDT $0.35 per base at 25 nm scale; no modified bases, standard desalting. V is any base except T	$9.10	870,000 (for typical yield of ~30 nanomoles)	$0.00001
PCR primers, 2 uM concentration	IDT; length of For = 18-20 bases, length Rev = 27	~$16	6,600 (If avg yield was 33 nanomoles)	$0.0024
VeriQuest FAST SYBR Green 2X qPCR mix	USB/Affymetrix	~$35 per mL	100	$0.35
			Cost per qPCR:	**$0.39**

Protocol: Before starting, make 10x reaction buffer containing ATP by mixing 150 µL of 10X reaction buffer provided with the poly A polymerase with 50 µL of 10 mM ATP also provided with the poly A polymerase.

Assemble 10 µL RT reactions according to Table 2 below. For processing multiple samples, scale up volumes as needed to make a Master Mix containing all components except sample RNA. See Note 1 for suggestion on reagent conservation when assembling Master Mixes. Input RNA may be total RNA or the small RNA enriched fraction. Total RNA and small RNA enriched RNA can be extracted using the BiooPure reagent (Bioo Scientific cat# 5301). Small RNA can also be extracted using the MiraZol™ Kit (Bioo Scientific cat# 5302) or the Next Prep Small RNA Isolation Kit (Bioo Scientific cat# 3812). RNA concentration is not critical and can vary over several logs. However, using higher volumes of input RNA can be problematic if inhibitors are present. The lower limit for input RNA to allow detection depends on the abundance of the specific microRNA targets, and can be determined empirically.

Table 2. Assembly of RT reactions

Input RNA	10X buffer with ATP	4dNTP mix, each at 1 mM concentration	RT primer at 10 µL concentration	Water	M-MLV Reverse Transcriptase, 200 units/µL	Poly A Polymerase, 5 units/µL
2 -5 µL	1 µL	1 µL	1 µL	To give final volume of 10 µL	0.4 µL	0.15 µL

- Incubate the reaction at 42˚C for one hour.
- Then incubate at 90˚C for 5 minutes to inactivate the reverse transcriptase.
- Then dilute the reaction by adding 80 µL of nuclease-free distilled water, vortex, pop-spin, and store at -20˚C or proceed to qPCR step.

Note, depending on abundance of the specific target microRNA(s), it may be possible to further dilute the cDNA reaction, or to make it less dilute to allow detection of rare targets.

qPCR step: For each 20 µL qPCR, use 3 µL of diluted RT reaction, 2 µL of [For + Rev] primers (each present at 2 µM concentration), 5 µL water, and 10 µL of 2x SYBR qPCR Mix. See Note 1 below for suggestion for conserving the qPCR Mix. See Note 2 for information on accessing free software for designing the Forward and Reverse PCR primers.

Run the samples on a real-time thermal cycler programmed according to the melting temperatures of the primers and the recommendations for the specific thermal cycler used. A typical program for qPCRs run on a BioRad 1Q5 real-time thermal cycler is:

a. initial denaturation hold step for 1 minute at 95˚C

b. 2-step cycle step consisting of 40 cycles of 95˚C for 1 minute followed by 58˚C for 30 seconds, with data acquisition at the end of each cycle

c. melt-curve analysis: 55˚C-90˚C in 0.5˚C increments, with 3 second dwell time at each temperature.

Note 1: Conservation of expensive reagents. When assembling Master Mixes, the volumes of components are typically scaled up slightly beyond the calculated volumes needed, in order to accommodate pipetting error and loss of reagent on the walls of the tubes and pipet tips. For example, the TaqMan MicroRNA Assay instruction manual from Life Technologies recommends adding 10% to 20% excess volume of each component to compensate for losses that occur during pipetting. Of course, this means that the actual number of assays that can be carried out per kit is reduced by 10-20%! Bioo Scientific suggests an alternative approach that avoids wasting expensive reaction components, such as poly A polymerase, Reverse Transcriptase, and qPCR mix. As an alternative to scaling up all components, simply scale up the inexpensive components such as water, 10x reaction buffers, or PCR primers. Scaling up these reagents by ~15% will typically provide sufficient overage while allowing the maximum number of assays to be carried out with the expensive limiting components.

Note 2: Software for design of Forward and Reverse qPCR primers. An algorithm for design of the PCR primers to detect any microRNA found in public databases such as miRBase using the described method has been developed by Dr. Peter Busk. The software is available for no cost and can be accessed at the following link:

http://tinyurl.com/qeo9a5o

Representative Data Showing Detection of microRNAs in Cell-free Fluids

RT-qPCR analysis of microRNAs using miR-specific qPCR method. Samples were from cell culture media from 293-T cells grown to high density in serum-free media. Low-speed spin was used to remove intact cells. Sample 30Filt is from 13 mL of media passed through a 30 nm pore size filter to capture exosomes, then the filter was removed and processed for RNA extraction using BiooPure RNA Extraction Reagent (Bioo Scientific cat #5301). Sample FT is 0.2 mL of flow-through from the 30 nm filter, extracted using 1 mL

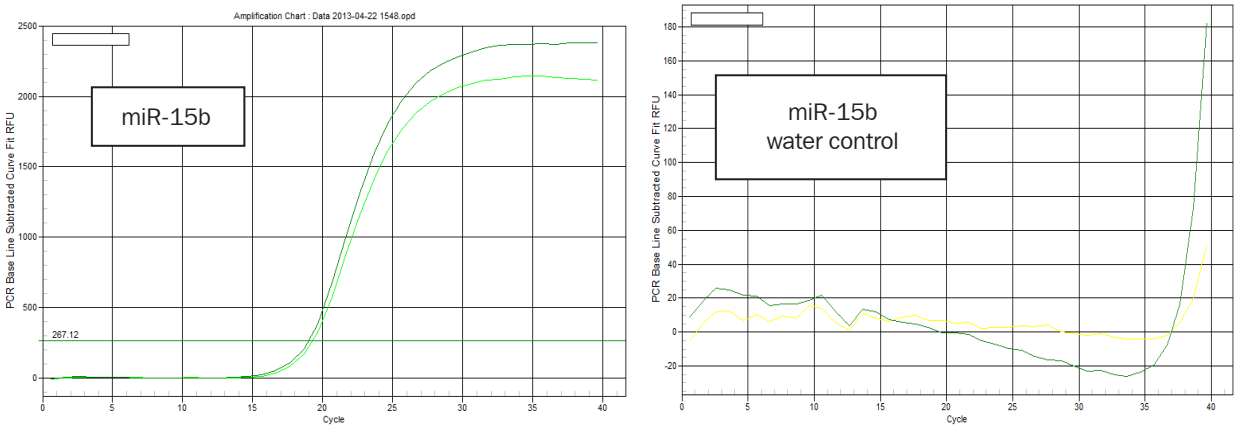
BiooPure. Sample EL30 is from material eluted from another 30 nm filter, used to filter 14 mL of the culture media, then eluted in 0.4 mL of water, and RNA then extracted from 0.25 mL of eluate. For all samples, the RNA pellets were resuspended in 20 µL and 5 µL was used as input for RT step. Sample Water Neg is using 5 µL of water as input for the RT.

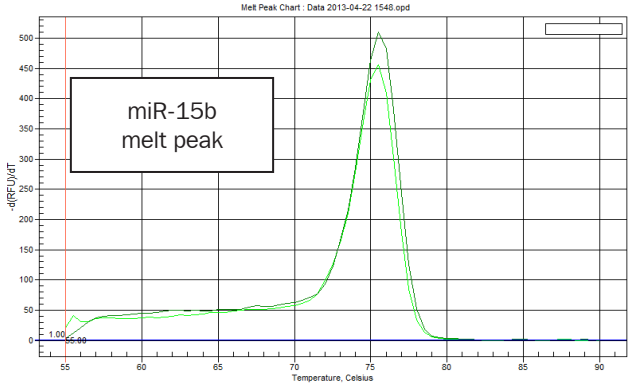
Table 3: Ct values of duplicates and average Ct

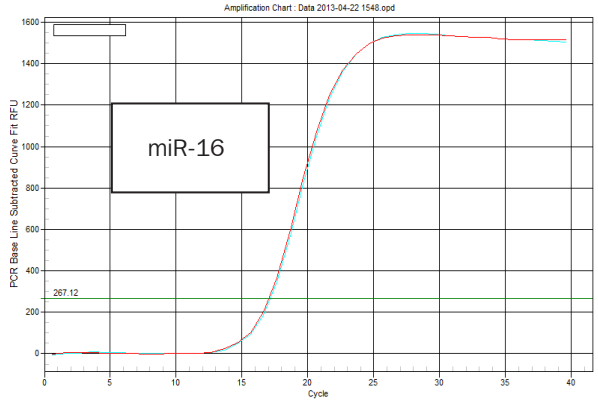
Target miR	30Filt	FT	EL30	Water neg
miR-15b	24.08/24.02/24.05	30.50/30.40/30.45	26.60/26.44/26.52	N/A, N/A
miR-16	21.60/21.48/21.54	27.04/26.95/27.00	22.60/22.61/22.60	N/A, N/A
RNU-6	25.03/25.02/25.03	31.94/32.57/32.25	25.43/25.63/25.53	N/A, N/A

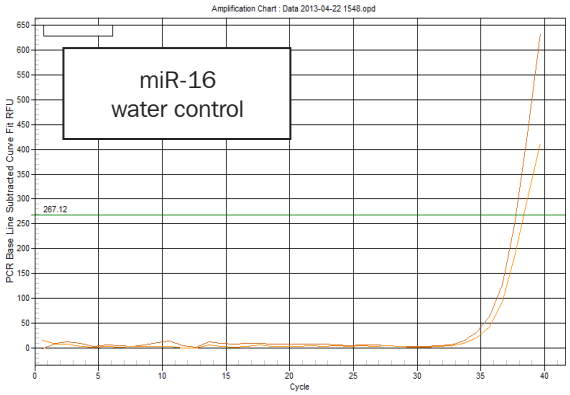
Representative RT-qPCR amplification plots and melt curves. Input RNA was extracted from particles captured on a 200 nm pore size filter from ~25 mL of cell-free fluid (293F cell culture media, serum-free). RNA was extracted from the filter using BiooPure Reagent and recovered in 50 µL, and 5 µL was used as input for the RT as described above. The RT was diluted to 90 µL and qPCR was performed using 3 µL of diluted RT in duplicate 20 µL reactions, with 10 µL of 2x qPCR SYBR Master mix, and primers specific for miR 15b, miR 16, and RNU6. Negative controls for qPCR had 5 µL of water instead of RT. The qPCR profile was as follows:

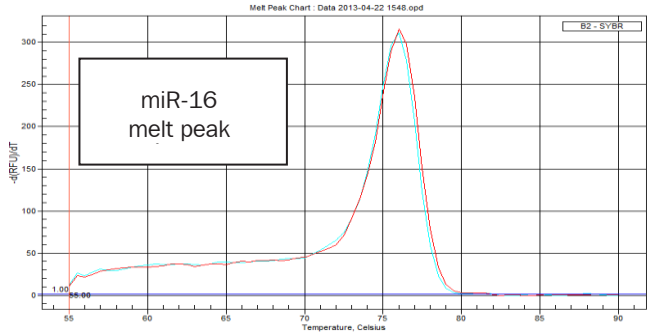
Amplification:
Hold 95˚ for 3 minutes
Cycle 40x: 95˚ for 10 seconds / 54˚ for 30 seconds
Melt curve: 55˚ - 90˚, 0.5˚ increments, 5 seconds dwell

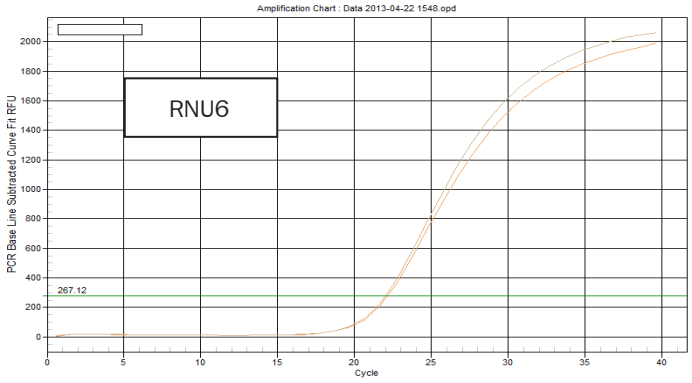


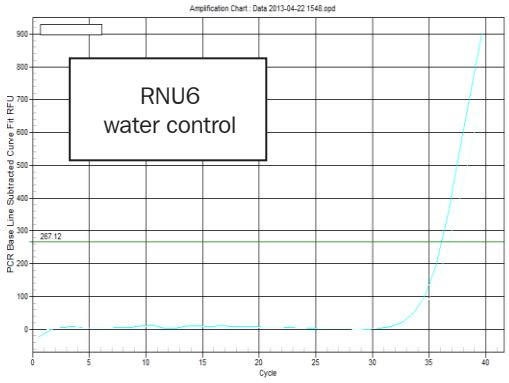


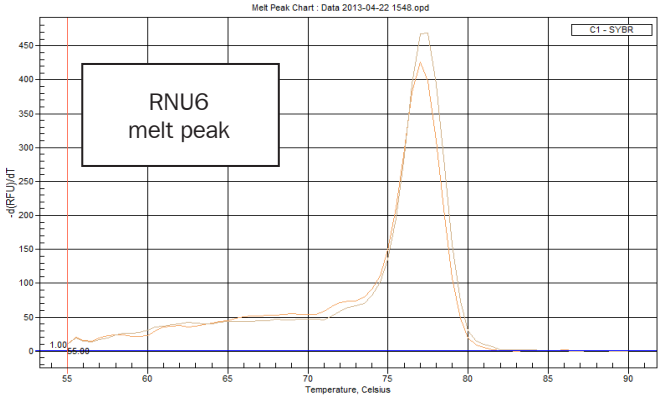

Amplification Chart : Data 2013-04-22 1548.opd
PCR Base Line Subtracted Curve Fit RFU
Cycle
267.12
miR-16


Amplification Chart : Data 2013-04-22 1548.opd
PCR Base Line Subtracted Curve Fit RFU
Cycle
267.12
miR-16
water control


Melt Peak Chart : Data 2013-04-22 1548.opd
B2 - SYBR
-d(RFU)/dT
Temperature, Celsius
1.00
55.00
miR-16
melt peak


Amplification Chart : Data 2013-04-22 1548.opd
PCR Base Line Subtracted Curve Fit RFU
Cycle
267.12
RNU6


Amplification Chart : Data 2013-04-22 1548.opd
PCR Base Line Subtracted Curve Fit RFU
Cycle
267.12
RNU6
water control


Melt Peak Chart : Data 2013-04-22 1548.opd
C1 - SYBR
-d(RFU)/dT
Temperature, Celsius
1.00
55.00
RNU6
melt peak

Cost Analysis of Several Methods for qPCR-based microRNA Detection

The cost per qPCR for the miR-specific qPCR method includes cost for poly A Polymerase, M-MLV Reverse transcriptase, dNTP mix, RT primer, qPCR primers, and 2x SYBR Green qPCR mix. The cost for these reagents is itemized in Table 1. Total cost for miR-specific qPCR method: $0.39

Cost per qPCR for Taqman microRNA from Life Technologies: $280 for custom, and $173 for inventory, target-specific RT primer and qPCR primers and probe, containing RT primer for 50 assays and qPCR primers and Taqman probe for 150 assays (so cost per assay is $5.60 or $3.46, since each target requires separate RT primer); $306 for cDNA synthesis kit (cat# 4366596, 200 rxns, "Taqman microRNA Rev Txn kit, so cost per RT is $1.53); $134 per 1.5 mL for 2x qPCR Mastermix (qPCRs are 20 µL, so cost per qPCR is $0.89 for qPCR mix). Cost for RT primer/PCR primers/Taqman probe + RT reaction + qPCR mix = $5.60 per RT or $3.46 per RT, including cost of qPCR primers and Taqman probe. Note that each microRNA target requires a specific RT primer, so cost is higher for looking at multiple targets in a single sample, compared to looking at the same target in multiple samples. Total cost for looking at the same target in 50 samples: $8.02 (for custom assays) or $5.88 (for microRNA assays in inventory). 15-fold – 21-fold higher than miR-specific qPCR method!

Cost per qPCR for Exiqon microRNA: $104 for primers for 200 rxns, $313 for cDNA kit for 8-64 rxns, $353 for 2.5 mL of 2xSYBR qPCR mix. Cost per assay = $1.41 per rxn for qPCR mix + $4.89/rxn for RT kit (if get 64 RTs) + $0.52 for primers. Total cost : $6.82. 17-fold higher than miR-specific qPCR method!

Cost per qPCR for Qiagen microRNA: miScript SYBR Green qPCR kit with universal Reverse primer, cat# 218073, cost is $437 for 200 rxns. miScript II RT Kit, cat#218161, cost is $439 for 50 rxns. Custom microRNA Forward primer, cat#218200, cost is $83.70 for 100 rxns. Cost per assay = $2.18 per rxn for qPCR mix and reverse primer, $8.78/rxn for RT, $0.83 per rxn for microRNA target-specific Forward primer. Total cost : $11.79 per qPCR per target. 30-fold higher than miR-specific qPCR method!

Conclusions

The miR-specific qPCR method is a cost-effective alternative to more expensive commercial products for qPCR-based microRNA analysis. Data presented in this article demonstrate use of microRNA-specific qPCR is sufficiently sensitive to allow detection of microRNAs, even in cell-free fluids. The availability of free software for designing the microRNA-specific PCR primers removes a previous obstacle to adopting the method, and will facilitate analysis of large panels of microRNA targets.

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