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Multiplexing RT-PCR for the detection of multiple miRNA species in small samples

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Abstract

MicroRNAs are short (~22 nucleotides), non-coding RNAs that play critical roles in gene regulation and may be used as rapid precise diagnostic indicators of early stages of cancer. The small size of these RNAs makes detection of multiple microRNA species in very small samples problematic. Here we investigate the parameters associated with multiplexing RT-PCR to obtain relative abundance profiles of multiple microRNAs in small sample sizes down to the amount of RNA found in a single cell.

Keywords: Multiplexed RT-PCR; miRNA; miRNA profiling; miRNA in small samples; Multiple primer reverse transcription

MicroRNAs (miRNAs) are small single-stranded RNAs, ~22 nucleotides long, that play important regulatory roles in plants and animals [1,2]. Studies in *Drosophila*, *Caenorhabditis*, and mammals have implicated miRNAs in a wide range of metabolic and developmental processes [3]. As potential clinical diagnostic tools miRNAs have been shown to be important and accurate determinants for many if not all cancers [4].

Over 320 miRNAs have been discovered in human and the numbers of miRNAs in this and other metazoans continue to grow (Sanger Center miRNA registry, www.sanger.ac.uk/cgi-bin/Rfam/mirna/browse.pl). Recently, an integrative approach combining bioinformatics predictions and microarray analysis indicated that the total number of human miRNAs may be in excess of 800 [5]. On the surface, microarray miRNA profiling is the obvious direct method to assay for multiple RNA species in a single reaction. Unfortunately, array hybridization methods employed to date require relatively large amounts of RNA, >1 μg quantities ([6–8], for examples). This amount of RNA is well above the RNA that one would isolate to answer some of the more important biological questions. For example, questions about early tumor formation or stem cell differentiation are best interrogated at the single or few cell level to avoid the problem of contamination with multiple non-target cell types.

Although real-time RT-PCR has been a gold standard for the quantification of mRNA, at first glance the direct use of this method would not appear to be readily applicable to miRNA quantification because miRNA is only 21–23 nucleotides long. To get around this apparent problem, Schmittgen et al. [9] adapted real-time RT-PCR to the longer precursor molecules of miRNA. However, this method has inherent assumptions about miRNA processing and presumed knowledge about the relationship between the amount of pre-miRNA and mature miRNA.

Two real-time PCR methods have been reported to determine the concentration of individual mature miRNAs. Shi and Chiang [10] treated 1 μg of RNA with polyA polymerase and then used RACE primers to add an adapter sequence to the reverse primer for subsequent real-time PCR with miRNA specific forward primers. Chen et al. [11] used stem-loop reverse primers with short, six nucleotide, protruding 3′ sequences that were complementary to 3′ miRNA to prime the RT reaction. Then, miRNA specific forward primers were used in conjunction with the

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loop sequences of the original reverse primer to do sensitive real-time PCR capable of detecting a particular miRNA in a single cell. Recently, Tang et al. [12] reported that a modified version of the stem-loop RT/real-time PCR procedure can be multiplexed to profile mouse miRNAs in single embryonic stem cells.

Here we examine the various steps of multiplexed, stem-loop RT/PCR for miRNA profiling and suggest primer and procedural designs that maximize the ability to detect multiple miRNAs under multiplexed reaction conditions.

Materials and methods

RNA and DNA. Total RNA samples from human lung and heart were obtained from Ambion Inc. Synthetic let-7a miRNA was purchased from Integrated DNA Technologies Inc. All DNA oligonucleotides were synthesized inhouse by Applied Biosystems.

Outline of procedure. Fig. 1 shows a graphic representation of the protocol of this paper. Steps 1 and 2 are multiplexed reactions with 48 or 190 sets of primers for 48 or 190 miRNAs. Step 1 reverse transcribes all the miRNAs in a single reaction and then step 2 PCR amplifies the cDNA products to provide enough sample for step 3. Step 3 is done as simultaneous, individual singleplex TaqMan® reactions in 384-well reaction plates to monitor the abundance of each miRNA after the multiplexed RT-PCRs. Sequences for primers and probes will be provided by the authors upon request.

Reverse transcription. Reverse transcription reactions of 5 μl contained: 0.5 μl of 10× cDNA Archiving kit buffer (Applied Biosystems), 0.335 μl MMLV reverse transcriptase (50 U/μl), 0.25 μl of 100 mM dNTP, 0.065 μl of AB RNase inhibitor 20 U/μl, 0.5 μl of 48- or 190-plex reverse primer, RP (50 nM each), 2 μl of human lung purified total RNA, and 1.35 μl H_2O. The reaction mixture was prepared by adding 2 μl of RNA sample to 3 μl of freshly prepared stock reaction mixture containing the remaining reaction ingredients for at least 10 reactions. The reaction was performed with the following incubation conditions: (20 °C/30 s—42 °C/30 s—50 °C/1 s) 60 cycles. The enzyme was subsequently inactivated by incubation at 85 °C for 5 min.

Pre-PCR amplification. The pre-real-time PCR mixture of 25 μl contained: 12.5 μl of 2× Universal Master Mix® with no UNG (Applied Biosystems), 5 μl of RT sample, 2.5 μl of 48- or 190-plex forward primer, FP, at 500 nM each, 1.25 μl of 100 μM universal reverse primer, UR, 1.25 μl of 5 U/μl AmpliTaq Gold®, 0.5 μl of 100 mM dNTP, 0.5 μl of 100 mM $MgCl_2$, and 1.5 μl dH_2O. The temperature profile for the reaction contained a 10 min incubation at 95 °C to activate Taq-GOLD®, a 55 °C incubation for 2 min, followed by 14 cycles of 95 °C for 1 s and 65 °C for 1 min.

Real-time PCR. The pre-amplified RT-PCR mixture was diluted to 100 μl by adding 75 μl H_2O into 25 μl of pre-amplified sample. The probes for each TaqMan® reaction consist of the 3′ 18 nucleotides of the RT-RP for each miRNA with the fluorescence dye, FAM at the 5′ end and a minor groove binder with non-fluorescence quencher, MGB, on the 3′ end.

Real-time reaction mixtures contained 5 μl of 2× Universal Master Mix® with no UNG (Applied Biosystems), 2 μl of a 5 μM FP + 1 μM TaqMan® probe mixture, 0.1 μl of 100 μM UR, 0.1 μl of 4× diluted pre-amplified RT-PCR sample, and 2.8 μl dH_2O. Real-time reaction mixtures were assembled by adding 2 μl of individual FP + TaqMan® probe to 8 μl of freshly prepared stock solution containing the rest of the real-time PCR reagents. Real-time PCR was performed on an AB 7900 HT Sequence Detection System in a 384-well plate format, with the temperature regime consisting of a hot start of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The real-time PCRs for each miRNA were run in duplicate.

Results and discussion

Many important studies or clinical assays require the determination of the abundance of multiple miRNAs from a single small sample. One approach to creating enough sample to assay each miRNA is to amplify each miRNA by multiplexed RT-PCR. For the procedure to work both the RT step and the PCR sample amplification step must be efficient. The difficulty of using RT-PCR directly on total RNA preparations is that the short miRNA sequence must be partitioned between two distinct sets of primers, those for reverse transcription and those for PCR amplification. Since overlap of the 3′ ends of these two primer sets can cause serious primer dimer problems for PCR, there are necessary length constraints on the complementary priming sequence. Therefore, primer design is crucial to the success of the method.

After extensive preliminary investigations with linear primers, the stem-loop structure of Chen et al. [11] was used to reduce spurious priming with larger RNA molecules. To evaluate the efficiency of RT priming as a function of priming sequence length, RT primers with 5–10 nucleotides of 3′ priming sequence for let-7a miRNA were evaluated for RT priming efficiency. Table 1 shows that priming efficiency, both against synthetic let-7a miRNA and total human lung RNA, increased rapidly with complementary sequence length to 8 nucleotides with less improvement thereafter. Similar priming efficiencies were observed when strictly linear RT primers were used (data not shown). Therefore, the primer design selected for this protocol differs from that of Chen et al. in that the RT priming complementary sequences are two nucleotides longer, which translates to an important reduction of about 4 C_ts of real-time PCR or 16 times as much cDNA. The effect of multiplexing the RT and PCRs with this design was studied at two levels, 48-plex (48 sets of miRNA primers) and 190-plex (190 sets of miRNA primers).

The pre-PCR amplification step (step 2, Fig. 1) is crucial to this procedure because it is necessary to generate enough material from small samples to perform individual

Table 1
Comparative reverse transcriptase efficiencies for stem-loop primers with 5, 6, 7, 8, 9, and 10 nucleotides complementary to the 3′ end of let-7a miRNA

5 nM RP	10 pM Let-7a	SD	10 ng HL	SD
RP-5-mer	25.75	0.26	26.68	0.97
RP-6-mer	19.82	0.11	19.99	1.07
RP-7-mer	18.10	0.42	18.34	0.64
RP-8-mer	15.44	0.22	16.22	0.65
RP-9-mer	14.37	0.25	14.25	0.40
RP-10-mer	13.42	0.10	13.77	0.86
No RP	31.13	0.65	29.92	0.49

Reverse transcription was performed on synthetic Let-7a RNA and total human lung (HL) RNA as described in the Material and methods. Pre-PCR was done for 14 cycles as described in Materials and methods. Real-time PCR was determined in singleplex as described in the Materials and methods.

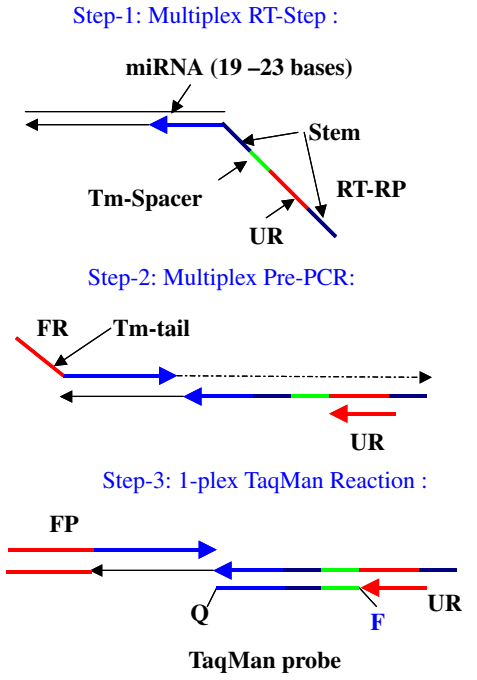

Fig. 1. A schematic representation of the 3 different steps for the multiplexed analysis of miRNA RT-PCR. Step 1 and step 2 are multiplexed RT and PCRs, respectively. The multiplexed forward primers, FP, contain 3′ sequences that correspond to the 5′ end of different miRNAs and 5′ sequences zip-coded to each RNA to increase the T_m of annealing above 65 °C. Step 3 illustrates singleplex real-time PCRs to assess the amount of miRNA specific cDNA. RT-RP is a miRNA specific RT reverse primer; FP is miRNA specific forward primer; UR is universal reverse primer for multiplex pre-PCR and singleplex real-time PCR assays. Each real-time PCR probe has a 5′ FAM dye label and a 3′ minor groove binder with non-fluorescent quenching activity (MGB). The T_m spacer is added to give probes T_m values above 60 °C.

real-time PCRs. However, for the procedure to work, each miRNA must be amplified with the same relative efficiency for each PCR cycle. To test this, cDNA from multiplexed RT reactions was amplified for 1, 5, 10, and 14 cycles of multiplexed PCR, and then the relative abundance of each miRNA was determined by individual real-time PCRs. Since the creation of an artificial mixture of synthesized miRNAs is prohibitively expensive, we used total RNA from human lung with the knowledge that some human miRNAs are not expressed in this tissue.

Figs. 2A and B show the C_t values of individual miRNAs for the 48- and 190-plexed reactions, respectively as correlation scatter plots with the C_t values for 10 cycles on the *Y*-axis and the C_t values of all amplifications on the *X*-axis. If the same relative efficiency of amplification for each miRNA is maintained throughout all cycles of amplification, the slopes of all the lines should be or approach 1, i.e., be parallel to the line for 10 cycles of PCR which must have a slope of 1 since it is a value plotted against itself. The calculated slopes for 1, 5, 10, and 14 cycles were 1.0 to one place of decimal for both 48- and 190-plex reactions, indicating that substantial pre-PCR amplification does not add significant bias to the relative concentrations of the miRNAs assayed.

To evaluate the dynamic range of this multiplexed RT-PCR method, total RNA was diluted from 100 ng to 10 pg and subjected to multiplex RT-PCR using 14 cycles of amplification for the pre-PCR. Fig. 3 shows the effect of total RNA input on the relative abundance of different miRNAs. For comparison the data are presented as correlation scatter plots with the C_t values for 10 ng RNA template on the *Y*-axis and the C_t values for 100 ng to 10 pg human lung total RNA samples on the *X*-axis for 48- and 190-plex reactions.

In these experiments, Applied Biosystems' AB 7900 HT Sequence Detection System detects single copy templates as those templates with a C_t of 37. However, because the miRNA was amplified with 14 cycles of PCR and then diluted 400× (a loss of 8.7 C_ts), the actual C_t value of single copy DNA is 31.3 (37 − 14 + 8.7). Therefore in assessing the validity of this protocol, we restricted our data analysis to miRNA data with C_t values less than 32 (Fig. 3). If the relative efficiencies of determining miRNA concentration are maintained throughout the dilution series, the slopes of lines representing different dilutions of RNA should be or approach 1, i.e., be parallel to the line for 10 ng RNA which must have a slope of 1 since it is a value plotted against itself. The calculated slopes for the lines through the 100 ng to 0.01 ng dilution plots are 1.0 to one place

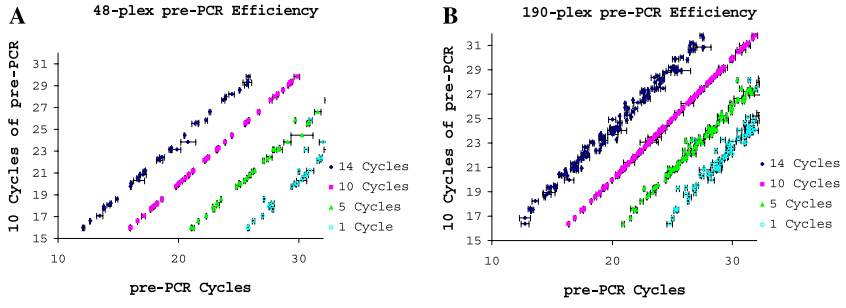

Fig. 2. The comparative efficiency of amplification of different miRNAs for different levels of PCR pre-amplification ("pre-PCR"). After multiplexed reverse transcription, separate PCRs were performed for 1, 5, 10, and 14 cycles, the C_t values of each miRNA were measured by singleplex real-time PCR. The results are plotted as correlation scatter plots with the C_t values for 10 cycles on the *Y*-axis and the C_t values of all amplifications on the *X*-axis. (A) 48-plex RT-PCR and (B) 190-plex RT-PCR.

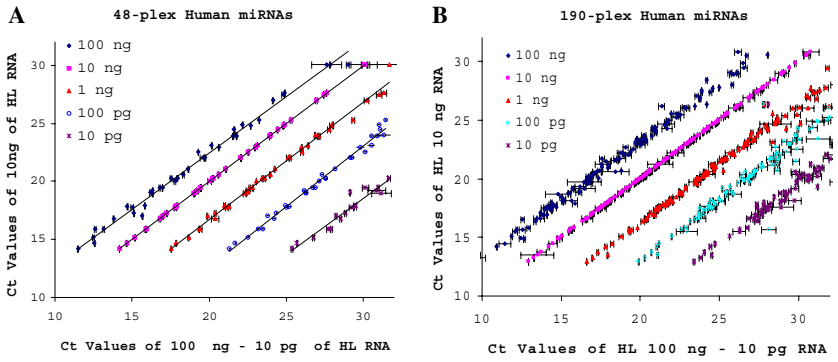

Fig. 3. Correlation scatter plots of the C_t values for different miRNAs in total lung RNA amounts that were decreased by 10-fold increments. (A) 48-plex RT-PCR and (B) 190-plex RT-PCR.

of decimal for both 48- and 190-plex (the worst slope was 0.96 for 0.01 ng 48-plex which had the fewest points on the plot). Since these values are very close to one, this experiment indicates that multiplexing miRNA detection reactions even to the 190-plex level should provide acceptable relative miRNA abundance profiles in very small sample sizes.

Since this study was on total human lung RNA, the presence of each of the 190 miRNAs in 10 ng of total human lung sample was assayed by RT real-time PCR using the same protocol configuration. When only one primer set was present, RT real-time PCR results indicate that 154 of the 190 miRNAs have at least 10 copies of cDNA and thus can be assessed with a substantial degree of accuracy without wide statistical variations from stochastic effects. When multiplex reactions were done on total heart RNA, an additional 27 miRNAs showed substantial reaction in the multiplex configuration (data not shown).

This study indicates that relative abundance profiles of multiple miRNAs can be generated for most miRNAs from very small samples. However, the study also shows that there are increasing effects of primer–primer interaction with an increase in multiplexing. This is seen in increased differences between the real-time PCR C_t value of a miRNA which was reverse transcribed and amplified for 14 cycles of PCR under singleplex conditions compared to the real-time PCR C_t value of a miRNA which was reverse transcribed and amplified for 14 cycles of PCR under multiplex conditions, Fig. 4. There is much more scatter in data points when the190-plex C_t value for each miRNA is plotted against its singleplex C_t value, Fig. 4B, than when 48-plex C_t values are plotted against singleplex values. These considerations mean that miRNA multiplexing should be treated the way microarray data have been treated, i.e., the results provide relative abundance profiles but that the actual amount of individual RNAs should be verified by singleplex real-time PCR [11].

Of course, many of the primer–primer interaction effects could be greatly reduced with a more indepth analysis of primer interactions. However, such studies would be exceedingly expensive and involve testing a massive number of primer combinations. With 326 known human miRNAs the work would be daunting for any laboratory or group of laboratories. On the other hand, robust data for most applications can be generated by applying the method of this paper to a moderate level of multiplexing, for example 48-plex, and fine-tuning primer mixtures for particular studies. In the clinical environment, such selected multiplexing might lead to valuable diagnostic procedures for very small biopsies.

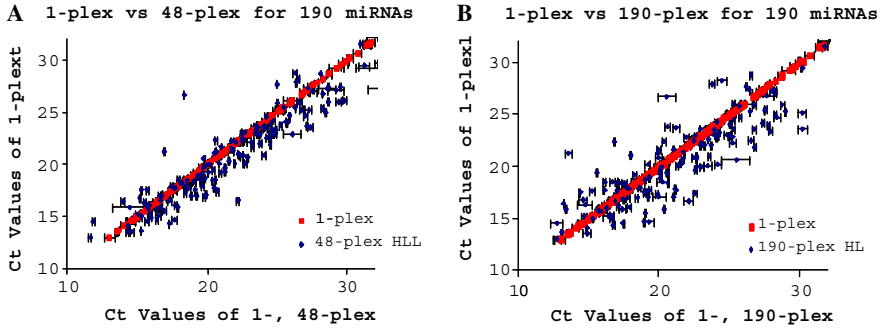

Fig. 4. The comparative effect of different levels of multiplexing both the RT and the pre-PCRs on the subsequent real-time PCR determinations of individual miRNAs. (A) Compares the real-time PCR values of the miRNAs of this study in singleplex reactions with four separate 48-plex reactions to cover all 190 miRNAs. (B) Compares the real-time PCR values of the miRNAs of this study in singleplex reactions with 190-plex reactions.

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