NEW METHODS AND TECHNOLOGIES

PCR-based expression analysis and identification of microRNAs

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ABSTRACT

microRNAs (miRNAs) are small RNAs that regulate translation and hence control a variety of cellular processes in metazoans. The quantitation and identification of miRNAs has been hampered by their small size and low abundance. Here we describe two robust PCR-based assays of miRNA expression based on the original cloning strategy. The non-quantitative PCR method allows detection and identification of miRNAs and we utilise this method in the discovery of a new miRNA (miR-532) in retinoic acid differentiated P19 cells. The second and quantitative method (QM-RT-PCR) is simple and accurate, and uses commonly available technology. Of particular interest is the specificity of this PCR-based technology compared to hybridisation-based methods including arrays and northern blotting. Here we have shown that a single base pair mismatch in the priming sequence results in a two order of magnitude reduction in the amplification of let-7f. These streamlined methods will complement previously described methods and will facilitate analysis of miRNA expression in rare cell populations where the amount of RNA is limited.

KEYWORDS: *microRNA, miR-532, RT-PCR, TaqMan, differentiation, hairpin RNA, let-7*

INTRODUCTION

miRNAs are endogenously encoded and produced small (~22 nucleotides) non-coding RNA molecules that have been implicated in the regulation of cellular differentiation, proliferation, apoptosis and metabolism. They are thought to mediate post-transcriptional gene regulation through interaction with target sequences in the mRNA. The result of this interaction may be translational repression (with partially mismatched targets in the 3′ untranslated region) or mRNA cleavage (with fully complementary targets in mRNA) (Kawasaki and Tiara, 2003; Moss et al, 1997; Olsen and Ambros, 1999; Wightman et al, 1993). The very broad phylogenetic distribution of miRNAs suggests that they have important functions, but most of these are yet to be discovered (Grad et al, 2003; Houbaviy et al, 2003; Lagos-Quintana et al, 2001; Lau et al, 2001; Lee and Ambros, 2001; Suh et al, 2004. Analysis of miRNA expression levels has been limited by a lack of sensitive and specific detection methods. To date, detection has typically relied on cloning, northern blotting or solid phase array analysis, which require that large quantities of RNA be available (Kawasaki and Tiara, 2003; Houbaviy et al, 2003; Calin et al, 2002; Chen et al, 2004; Krichevsky et al, 2003; Lee et al, 2003; Thomson et al, 2004). Northern blotting is currently the gold standard for miRNA detection (Ambros et al, 2003) but is also limited by the difficulties of achieving stringent hybridization conditions with these very small molecules. Recently, an elaborate modified Invader® assay (Third Wave Technologies, Inc) (Allawi et al, 2004) and an RNA-primed, array-based Klenow enzyme assay (RAKE) (Nelson et al, 2004) were reported to quantify miRNA levels. In addition, a quantitative RT-PCR method for the amplification of pre-miRNAs has been developed (Schmittgen et al, 2004). Additional methods for miRNA identification and quantification will provide flexibility and facilitate research in this emerging

field. We sought simple and robust methods to detect, quantify, and distinguish miRNAs, particularly when cellular RNA is limited.

MATERIALS AND METHODS

Cell culture, differentiation and RNA extraction

HeLa cells were cultured in DMEM supplemented with 10% (v/v) FCS (Sigma, St Louis, Missouri) and P19 mouse embryonic carcinoma cells were cultured in DMEM supplemented with 5% (v/v) FCS, at 37°C in a humidified atmosphere containing 5% (v/v) CO_2. The human leukemic cell line HL-60 was obtained from the American Type Culture Collection and maintained in RPMI-1640 medium containing L-glutamine supplemented with 10% (v/v) fetal bovine serum (Gibco, Invitrogen, California, USA) and penicillin/streptomycin (1000U, Gibco) at 37°C in a humidified atmosphere containing 5% (v/v) CO_2.

HL-60 differentiation experiments were performed using 3.5 x 10^6 cells in 10ml of medium. Monocyte-macrophage differentiation was induced by the addition of 12-O-tetradecanoylphorbol-13-acetate (TPA; 10^{-7} M, Sigma, St Louis, MO, USA) to the cultures for up to 5 days. Cells were characterised before and after differentiation induction by flow cytometry, and morphologically with May-Giemsa-Grunwald staining of cells centrifuged onto slides by cytospin (Shandon, Pittsburgh, PA, USA; 350 rpm, 10 min).

P19 differentiation experiments were performed in bacterial culture dishes to prevent cell adhesion and encourage the formation of embryoid bodies. Cells were plated in DMEM supplemented with 5% (v/v) FCS and retinoic acid (0.5 μM) for two lots of 48 hr (plates were changed at 48 hr to remove adherent cells). Total RNA was extracted using Trizol according to the manufacturer's instructions (Gibco Life Technologies), suspended in DEPC-treated water and stored at -70°C. For QM-RT-PCR of HL-60 cells in Figure 3B, RNA was isolated using the RNeasy Mini Kit (a procedure that selectively excludes small RNAs of < 200 nucleotides (RNeasy Mini Handbook, Qiagen)).

Adaptors and primers

The primers used in this work are listed in Table 1. To amplify miRNAs, a 3′ RNA-DNA adaptor was ligated to total RNA (Figure 1). Adaptor-50 or adaptor-64 was used for miRNA-RT-PCR and adaptor-Q was used for QM-RT-PCR. To minimize non-specific PCR amplification, the adaptors were designed to be non-homologous to any known genes, as determined by Blast homology searches (www.ncbi.nlm.nih.gov/BLAST). To amplify miRNAs or mRNAs, 5′ sequence specific primers were used in conjunction with 3′ primers complementary to the adaptor sequence. The expression of β-actin mRNA, detected using gene-specific forward and reverse primers, was used as a positive control. The primers used for sequencing were T7 or pGEM-Trevseq.

RESULTS AND DISCUSSION

The first method (Figure 1) is a non-quantitative but extremely sensitive reverse transcription polymerase chain reaction (RT-PCR) assay. This miRNA-RT-PCR first requires ligation of a DNA/RNA hybrid oligonucleotide adaptor to total RNA, as originally described for cloning of small RNAs (Elbashir et al, 2001; Lau et al 2001). The subsequent PCR reaction uses a 5′ primer that is complementary to ~15 nucleotides at the 5′ end of the reverse transcribed target miRNA. The 3′ primer is complementary to the adaptor (Figure 1A). This design leaves ~7 'unconstrained' nucleotides (depending on the length of the miRNA) at the 3′ end of the specific target miRNA. This portion of the amplified fragment is not determined by either of the primers and so may be used to confirm the identity of the amplified miRNA, by cloning and sequencing the PCR product. This improves on published methods by ligating only one universal adaptor to the 3′ end of the miRNA (Grad et al, 2003; Lim et al, 2003; Meister et al, 2004). We tested the ability of miRNA-RT-PCR to detect let-7a in HeLa cells, where its expression has been demonstrated by cloning and northern blotting (Lagos-Quintana et al, 2001). miRNA-RT-PCR produced the predicted 94 base pair fragment which includes the adaptor sequence (Figure 1B). The PCR product was cloned and sequenced; all of eleven sequenced clones were identical to let-7a, although three either lacked the 3′ thymidine or contained an additional thymidine at the 3′ end (data not shown). The let-7a primer should also amplify let-7b and let-7c, as the three miRNAs differ only in their six 3′ bases. let-7b and let-7c were not represented in the sequenced clones, which may indicate that HeLa cells do not express let-7b and let7c, or that they are at least ten fold less abundant than let-7a, consistent with RAKE microarray data (Nelson et al, 2004).

Table 1. Oligonucleotide sequences (p, phosphate; f, forward; r, reverse; uppercase, RNA; lowercase, DNA; X, inverted thymidine)

Name	Sequence (5' to 3')
adaptor-64	pUUUcactagcacgagctcgctacgctcactactcggcat tatgtacgctaactaactcgtggtcX
adaptor-50	pUUUcactattacgagtgactactcggcatcgtgcgaacc gcatccttctcX
adaptor-Q	pUUUacggctcaagtgagcgtagcgagctcgtgctagtga tatgtacgctaactaactcgtggtcX
RT for adaptor-64/Q	gaccacgagttagttagcgtacat
RT for adaptor-50	cgcggatccgagaaggatgcggtt
let-7f primer	ccggaattctgaggtagtagattg
let7-a/b/c primer	ggctgatatgaggtagtaggttgt
miR-532 primer	ccggaattccatgccttgagtgta
miR-19 primer	gactgacatgtgcaaatctatgc
miR-21 primer	cgtgacgttagcttatcagactg
GAPDH-f primer	tgcaccaccaactgcttagc
GAPDH-r primer	ggcatggactgtggtcatgag
TaqMan for adaptor Q	*fam*-tcactagcacgagctcgctacgctcact-*tamra*
TaqMan for GAPDH	*fam*-cctggccaaggtcatccatgacaactt-*tamra*
PGEM-Trevseq primer	ttaggcaccccaggctttacac
T7 primer	taatacgactcactatagggc

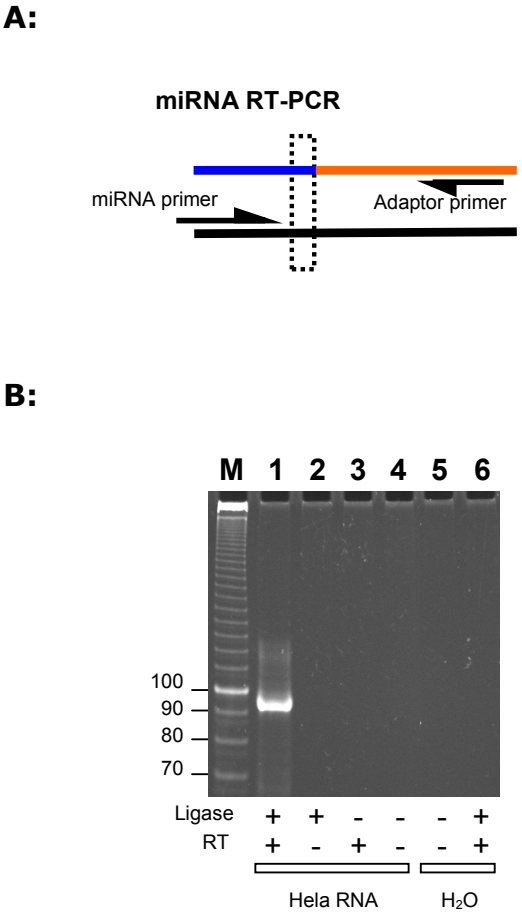


Figure 1. (A) The miRNA-RT-PCR method, showing the ligated miRNA (blue) and adaptor (orange) and the PCR primers. The dotted box represents the target sequence not determined by the primer. **(B)** Ethidium bromide stained polyacrylamide gel of let-7a/b/c specific miRNA-RT-PCR products assayed from HeLa cell total RNA and using adaptor-64 (Table 1) to confirm the reaction specificity. Lane 1 contains all amplification reagents; lanes 2-6 are controls for ligation of the adaptor (ligase), reverse transcription (RT) and template requirement (H_2O). M, molecular weight markers. **(C)** Decreasing amounts of HeLa cell total RNA assayed for let-7a/b/c using miRNA-RT-PCR. Amounts of total RNA used in the adaptor ligation were 5ng (lane 1), 0.5ng (lane 2), total RNA purified from 200 HeLa cells (lane 3), no RNA control (lane 4).

We tested the sensitivity of miRNA-RT-PCR by diluting total RNA from HeLa cells and assaying for let-7a. As little as 0.5 ng of total RNA from HeLa cells was sufficient to yield the let-7a product (Figure 1C). This indicates that miRNA-RT-PCR is at least 100-fold more sensitive than a commercial solution hybridization assay [*mir*Vana™ miRNA Detection Kit, Ambion] and the modified Invader assay (Allawi et al, 2004), both of which are more sensitive than the current 'gold standard' northern blotting (Ambros et al, 2003). To further assess sensitivity we used total RNA isolated from 200 HeLa cells and obtained the let-7a product (Figure 1C). This indicates that miRNA-RT-PCR may be useful in detecting miRNA expression in rare cell types, or when total RNA is limiting, such as after laser microdissection.

Another application for the miRNA-RT-PCR method is to verify the expression of newly-cloned miRNAs (Figure 2). Newly- cloned sequences, or miRNAs predicted *in silico*, require evidence that they are actually present in some cell type before they can be classed as a true miRNA (Ambros et al, 2003). We cloned a novel 22 nt miRNA, miR-532 (sequence: CATGCCTTGAGTGTAGGACCGT) from retinoic acid differentiated P19 embryonic carcinoma cells, but could not detect its expression in any tissue by northern blot analysis (data not shown). We assayed total RNA from HeLa cells using miRNA-RT-PCR with a 5′ primer specific to this newly identified miRNA, and

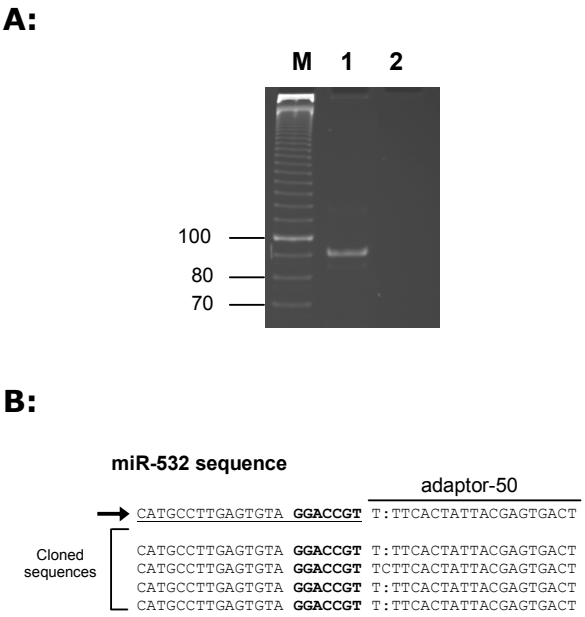
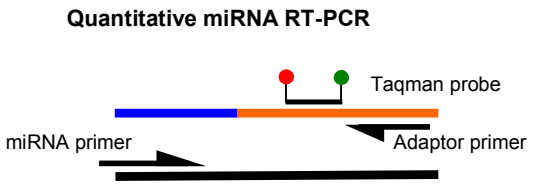
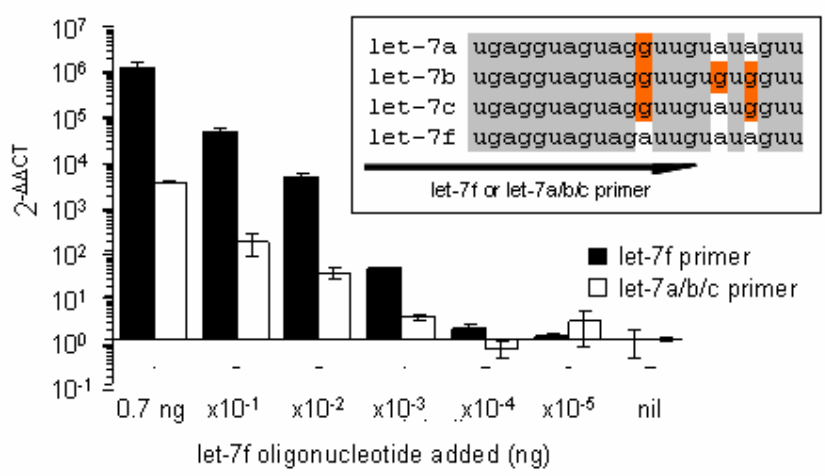


Figure 2. Characterisation of miR-532 expression using miRNA-RT-PCR. **(A)** miRNA-RT-PCR of total HeLa cell RNA using the 50 nt adaptor (adaptor-50) and a 5` primer specific for miR-532 resulted in a PCR product of the expected size (lane 1). A no template control is shown (lane 2), as visualised by gel electrophoresis. **(B)** Amplification of miR-532 was confirmed by sequencing four clones obtained from the purified PCR product (Expected sequence arrowed, miRNA derived sequence underlined, adaptor-50 derived sequence indicated, unconstrained nucleotides bold in all sequences shown).

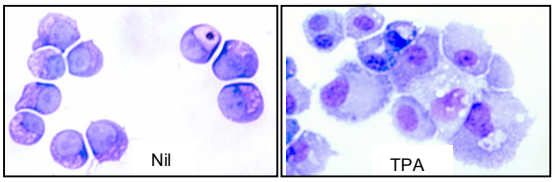
A:



B:



C:



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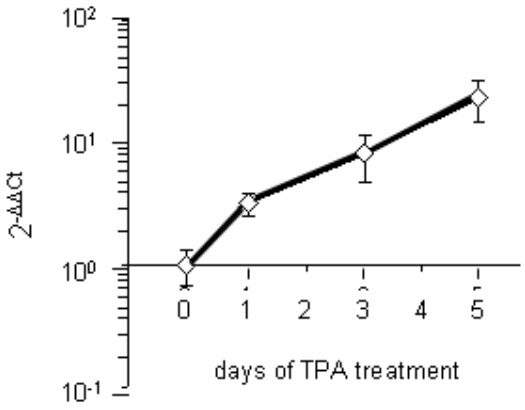

Figure 3. (A) The QM-RT-PCR method, showing the ligated miRNA (blue) and adaptor-Q (orange), the PCR primers, and the TaqMan probe. The TaqMan probe is complementary to the adaptor. **(B)** Amplification of let-7f is concentration dependent and specific. Amplification of 10-fold dilutions (starting from 0.7 ng) of synthetic let-7f, in HL-60 RNA depleted of small RNAs (1000 ng). Amplifications used either a let-7f primer (black columns) or a let7a/b/c primer (white columns). The rightmost column (nil) contains no synthetic let-7f. All experiments were performed in triplicate at least thrice, and error bars represent standard deviation from the mean. Inset shows aligned sequences of let-7-a/b/c/f, with identity indicated in grey and differences between let-7f and let-7a/b/c indicated in orange. **(C)** TPA-induced monocyte/macrophage differentiation of HL-60 cells. HL-60 cells were incubated with 10^{-7}M TPA for 5 days, spun onto slides and stained with May-Grunwald Giemsa for morphological analysis (100x). Untreated (Nil), 5 days TPA (TPA). **(D)** QM-RT-PCR of miR-21 during TPA-induced differentiation over 5 days. Error bars indicate standard deviation.
miRNAs that have 16 nucleotides of 5′ sequence identity, such as let-7a/b/c.

recovered a PCR product of the correct size (Figure 2A). Sequencing of four clones demonstrated that in each the seven unconstrained nucleotides matched the sequence of the cloned miRNA (Figure 2B), thereby confirming expression of this novel miRNA. The genomic sequence from which this miRNA sequence is derived is predicted to fold into a typical miRNA precursor hairpin structure in both the mouse and human genomes (data not shown). The miRNA-532 sequence is highly conserved in mammals (data not shown) and is located ~400nt upstream of miR-188. The proximity of miR-532 to miR-188 suggests they are processed from a common pri-miRNA transcript. These data demonstrate that miRNA-RT-PCR can be used to validate novel miRNAs and will be of particular importance as a means of experimentally confirming computationally predicted miRNAs (Xie et al, 2005).

Although it is extremely sensitive, miRNA-RT-PCR does not accurately measure or compare miRNA expression levels. To address this limitation, we incorporated a single TaqMan probe and real time PCR technology to create quantitative miRNA (QM)-RT-PCR. The TaqMan probe is derived from a portion of the adaptor and is complementary to the adaptor sequence (Figure 3A). Another TaqMan quantative RT-PCR based method using a self annealing 3' primer for miRNA detection will be released commercially, however, this method lacks utility in miRNA identification because all bases in the amplified miRNA are constrained by primer sequences (Applied Biosystems, Asia Pacific Advanced Genomics Symposium, 2005). As such sequencing of the product would not provide information on the specific target sequence(s) amplified.

We have applied QM-RT-PCR to many different miRNAs in various cell lines; here we demonstrate the key features of the method using let-7f as an informative example. 10-fold serial dilutions of synthetic let-7f were added to a fixed amount of HL-60 cell total RNA depleted of small (<200nt) RNAs. Following ligation and RT, amplification of let-7f was concentration dependent (Figure 3B). A relative measure ($2^{-\Delta\Delta Ct}$) of miRNA level was made by incorporating a reference target for all samples (Bustin, 2000), in this case GAPDH mRNA. Expression of miR-19, a miRNA with little similarity to let-7f, was not detectable in the same experiment (data not shown). To test the specificity of QM-RT-PCR, amplification was performed with an identical reaction mix in which the let-7f primer was replaced by a primer of the same annealing temperature but with a one base pair mismatch in the 15 5' priming nucleotides (sequence homologous to let-7a, let-7b and let-7c). All three let-7a/b/c sequences are identical in their 16 5′ nucleotides but differ from let-7f by a single base change (Figure 3B box). Use of the mismatched primer reduced amplification by greater than two orders of magnitude, confirming relative specificity under these challenging conditions (Figure 3B). We believe that such a stringent test of specificity has not been previously performed in this way and that the specificity of all arrays including northern blotting and solid phase methods has not been critically tested, as argued elsewhere (Nelson et al, 2004). Nevertheless, QM-RT-PCR would be limited in its ability to distinguish between the very few miRNAs that have 16 nucleotides of 5' sequence identity, such as let-7a/b/c.

To assess the utility of QM-RT-PCR in measuring the temporal regulation of miRNA expression, we examined miRNA miR-21 expression during TPA-induced differentiation to the macrophage lineage in HL-60 cells (Figure 3C) (Kasashima et al, 2004). Kasashima et al. reported a 13-fold increase in miR-21 after 2 days of TPA, measuring abundance by the cumbersome method of counting miR-21 clones. QM-RT-PCR measured an increase in miR-21 expression that exceeded 20-fold after five days (Figure 3D).

CONCLUSIONS

These two methods provide important additions to the tools available for the study of miRNA. miRNA-RT-PCR is a sensitive miRNA detection method that can be used to confirm the expression and identity of newly cloned miRNAs and uses generally available tools. It is likely this method will find immediate utility in confirming computationally predicted miRNAs. QM-RT-PCR is sensitive, quantitative, and economical: only one universal adaptor and one universal TaqMan probe allows the detection of any miRNA, and the specificity of the reaction for a unique miRNA is determined entirely by the 5′ miRNA specific oligonucleotide. This method is therefore well suited to high-throughput strategies for precise quantitation of miRNAs. Clearly, QM-RT-PCR provides a new tool to study temporal patterns of specific miRNA expression in development and cellular differentiation and will complement microarray expression profiling (Nelson et al, 2004; Thomson et al, 2004).

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STATEMENT OF COMPETING INTERESTS

The authors declared no competing interests.

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