MicroRNA Quantitation by RT-PCR

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ABSTRACT

A new miRNA quantitation method has been developed using stem-loop primers for reverse transcription (RT) followed by a real-time TaqMan® probe. A total of 240 miRNA assays were designed and tested using as little as 0.03 ng total RNA or 2-3 heat-treated cells. The C_T values correlated (R^2 = 0.999) to the copy number over seven orders of magnitude. The expression of miRNAs varied greatly from few to 32,000 copies per cell in mouse tissues. Presence of genomic DNA did not affect the miRNA quantitation. The assays discriminated between two miRNAs that differed by as little as a single nucleotide, and between mature miRNAs and their precursors. This method allows accurate and sensitive miRNA expression profiling and identifies potential miRNA markers specific to tissues or diseases.

INTRODUCTION

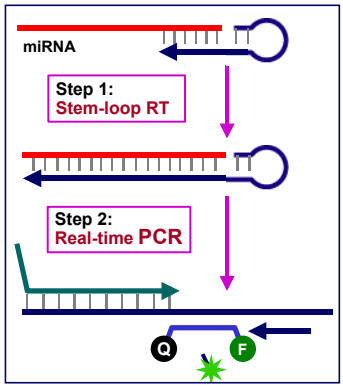
MicroRNAs are endogenous RNAs of ~22 nucleotides that play important regulatory roles in animals & plants by targeting mRNAs for cleavage or translational repression (1). More than 700 miRNAs have been identified across species. Their expression levels vary greatly among species and tissues (2). Low abundant miRNAs have been difficult to detect based on current technologies such as cloning, Northern hybridization (3), and the modified Invader® assay (4). Here, we present a new real-time quantitation method termed looped-primer RT-PCR for accurate and sensitive detection of miRNAs as well as other non-coding RNA (ncRNA) molecules.

MATERIALS & METHODS

miRNA targets: A total of 240 miRNA targets designed, mostly from human.
Tissue RNA samples: Ten human & mouse RNA samples were purchased from Ambion.
Cells & lysates: Six cell lines including HeLa, and 3T3 etc. were cultured to obtain cells and lysates using AB's lysis kits.
miRNA purification: miRNA was purified from cultured cells using *mir*Vana™ miRNA Isolation Kit (Ambion).
Northern: Solution hybridization-based Northern analysis was carried out using *mir*Vana™ miRNA Detection Kit (Ambion).
RT-PCR: The assay includes two steps, RT and PCR (Figure 1). RT reactions containing RNA samples, looped-primers, 1X buffer, reverse transcriptase, and RNase inhibitor were incubated for 30 min each, at 16°C and at 42°C. Real-time PCR was performed on an Applied Biosystems 7900HT Real-Time PCR System.
Data analysis: The copy number per cell was estimated based on the standard curve of synthetic lin-4 miRNA.

RESULTS

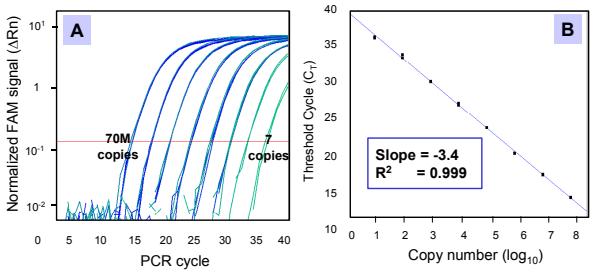
Figure 1. Assay scheme



Step 1. Stem-loop RT: stem-loop RT primers are annealed to miRNA targets and extended in the presence of reverse transcriptase.

Step 2. Real-time PCR: microRNA-specific forward primer, TaqMan® probe, and reverse primer are used for PCR reactions. Quantitation of miRNAs is estimated based on measured C_T values.

Figure 2. Quantitation of synthetic lin-4 miRNA



(A) Amplification plot of synthetic lin-4 miRNA over 7-logs. (B) Standard curve for lin-4 miRNA. C_T values were plotted against copy number.

Table 1. Mouse miRNA expression body map

	Copy number per cell*							
miRNA ID	Brain	Heart	Liver	Lung	Thymus	Ovary	Embryo	Average
let-7a	2010	1420	700	2390	1420	3120	1050	1730
miR-16	10240	13520	3890	22080	32090	11100	5210	14020
miR-20	70	300	130	580	1990	420	620	590
miR-21	670	2540	4450	7970	3550	5310	390	3550
miR-22	290	1020	310	590	130	560	40	420
miR-26a	7470	4360	3240	10680	2160	6880	1390	5170
miR-29a	4410	1040	730	7740	790	2950	20	2530
miR-30a	120	160	70	370	20	130	40	130
miR-34a	1240	140	90	430	200	490	60	380
miR-200b	20	1	10	210	40	130	30	60
miR-323	80	0	0	0	0	0	30	20
miR-324-5p	270	30	10	100	50	80	150	100
Average	2240	2040	1140	4430	3540	2600	750	**2400**

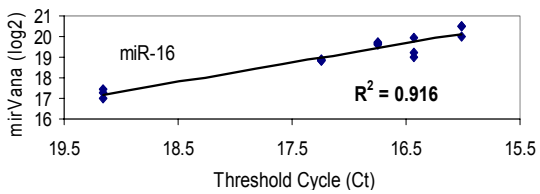
* Copy number per cell is estimated based on standard curve of lin-4 synthetic miRNA assuming 15 pg total RNA/cell.

Table 2. C_T comparisons of miRNA quantitation using whole cells, lysates, & purified RNA

miRNA source	mir-16	mir-21	mir-29	mir-30a	mir-200b	mir-323	Mean
GuHCl lysate	25.2	24.2	27.9	32.2	40.0	34.7	30.7
Low-salt lysate	22.3	22.0	26.5	28.9	40.0	31.1	28.5
Heat-treated cells	22.3	21.3	27.2	26.1	40.0	30.1	27.8
Purified miRNA	24.9	24.7	27.4	30.7	40.0	31.8	29.9

* A total of 2,000 HeLa cells or equivalent used in RT reaction.

Figure 3. Comparison of RT-PCR to Northern



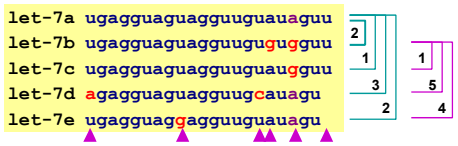
* Five mouse tissues were used. Total RNA input: 5 µg for Northern.

Table 3. Differentiation between mature and precursor forms of miRNA by C_T values

ID	Synthetic target (Copy No.)		TaqMan miRNA Assay (C_T)
	Mature miRNA	Precursor	
miR-26b	1.5×10^8	0	16.5
	0	1.5×10^8	27.4
	0	0	ND
let-7a	1.5×10^8	0	16.5
	0	1.5×10^8	29.5
	0	0	ND

Table 4. Discrimination of miRNA assays

miRNA assay	Synthetic miRNA target					Relative detection (%) *
	let-7a	let-7b	let-7c	let-7d	let-7e	
let-7a	100	0.3	3.7	0.0	0.0	
let-7b	0.0	100	0.3	0.0	0.0	
let-7c	0.0	2.5	100	0.1	0.0	
let-7d	0.1	0.0	0.0	100	0.0	
let-7e	0.0	0.0	0.0	0.0	100	



* Relative detection (%) calculated based on C_T difference between perfectly matched and mismatched assays.

Table 5. Effect of background genomic DNA on miRNA quantitation

	C_T		ΔC_T
gDNA (ng)	0	5	(0 vs 5)
let-7a	17.9	17.9	0.0
lin-4	33.7	33.8	0.1
miR-30a	22.5	22.5	0.0
miR-7	26.7	26.7	0.0
miR-107	19.6	19.6	0.1
miR-159	35.0	35.2	0.2
miR-124	31.9	32.3	0.4
miR-210	21.6	21.7	0.1
Average	26.1	26.2	**0.1**

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TRADEMARKS/LICENSING

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