MicroRNA Quantitation By Looped RT-PCR

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ABSTRACT

A new miRNA quantitation method has been developed using looped reverse transcription (RT) followed by real-time PCR. A total of 123 miRNA assays were designed and tested using 0.03-100 ng total RNA, as well as 100-10,000 lysed or nonlysed cultured cells. C_T values correlated ($R^2 = 0.998$) to the copy number over six orders of magnitude. miRNA expression varies greatly from 0 to 32,091 copies/cell in mouse. The assay discriminated between two miRNAs that differ by up to 1 nt, and between precursor and mature miRNAs. It will allow for quickly establishing accurate and sensitive miRNA expression profiles and identifying miRNA markers specific to tissues or diseases.

INTRODUCTION

MicroRNAs are endogenous RNAs of ~22 nucleotides that can play important regulatory roles in animals & plants by targeting mRNAs for cleavage or translational repression (1). Less than 1,000 unique 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, sequencing, Northern hybridization (3), and the modified Invader assay (4). Here, we present a new real-time quantitation method termed looped RT-PCR for accurate and sensitive detection of miRNAs.

MATERIALS AND METHODS

miRNA targets: 126 miRNA targets designed.
Tissue RNA samples: 10 human and mouse RNA samples were purchased from Ambion.
Cells and lysate: Six cell lines were cultured to obtain cells and lysate 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.
Looped RT-PCR: The assay includes two steps, RT and PCR. Details see Fig. 1.
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 Looped RT: Looped RT primers are annealed to miRNA targets and extended by reverse transcriptase.

Step 2 Real-time PCR: MicroRNA-specific forward primer, TaqMan® probe, and universal reverse primer are used for PCR reactions. Quantitation of a miRNA is determined from C_T values.
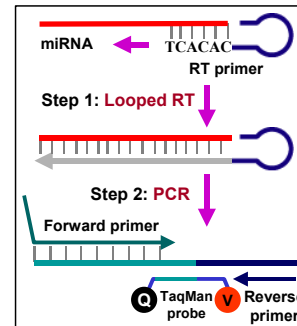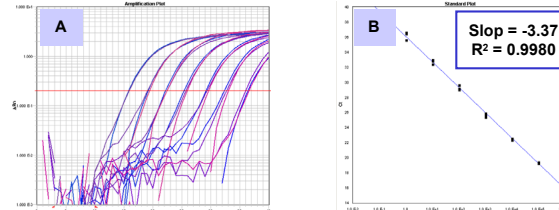
Figure 2. Quantitation of synthetic lin-4 miRNA

Slop = -3.37
$R^2 = 0.9980$

(A) Amplification plot of synthetic lin-4 miRNA over 6-logs in .2 μg UHR total RNA.
(B) Standard curve for lin-4 miRNA. C_T values were plotted against copy number.

Table 1. Mouse miRNA expression body map

miRNA ID	Brain	Heart	Liver	Lung	Thymus	Ovary	Embryo	Average
let-7a	2014	1421	698	2387	1419	3124	1050	1730
miR-16	10235	13524	3892	22080	32091	11097	5211	14019
miR-20	71	297	132	581	1992	418	616	587
miR-21	670	2535	4445	7969	3553	5307	388	3552
miR-22	294	1023	305	590	134	564	39.0	421
miR-26a	7465	4358	3244	10683	2157	6882	1386	5168
miR-29	4409	1041	731	7745	793	2949	17.0	2526
miR-30a	118	162	71	366	16.0	133	40.0	129
miR-34	1242	142	85	429	200	485	55.0	377
miR-200b	17.0	1.0	7.0	208	44.0	126	28.0	62
miR-323	76	0.0	0.0	0.0	0.0	0.0	32.0	15
miR-324-5	273	34.0	14.0	100	50.0	80	145	99
Average	2240	2045	1135	4428	3537	2597	751	2391

Copy number per cell

Table 2. miRNA quantitation of cells, lysate, & 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
Gold lysate	22.3	22.0	26.5	28.9	40.0	31.1	28.5
Cultured 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

Figure 3. Comparison of looped RT-PCR to Northern results

miR-16	Brain	Heart	Liver	Lung	Thymus	Ovary	Embryo
Northern blot							
RT-PCR (Copies/cell)	10235	13524	3892	22080	32091	11097	5211

Table 3. Differentiation of mature and precursor forms of miRNA (C_T)

Mouse RNA (ng)	miRNA (fM)	Precursor (fM)	Assay specific for miRNA*	Assay specific for Precursor
let-7a				
0	0	0	40.0	40.0
0	100	0	24.2	40.0
0	0	100	35.0	25.0
10	0	0	19.1	40.0
miR-26b				
0	0	0	40.0	40.0
0	100	0	23.1	40.0
0	0	100	32.9	25.7
10	0	0	20.5	28.0

Table 4. Discrimination of miRNAs that differ by up to 1 nt

miRNA assay		let-7a	let-7b	let-7c	let-7d	let-7e	let-7f	
	let-7a	28.8	37.3	32.6	39.4	40.0	34.0	Ct
	let-7b	40.0	27.3	32.2	40.0	40.0	40.0	
	let-7c	37.0	31.6	27.1	37.3	40.0	40.0	
	let-7d	40.0	40.0	40.0	27.4	40.0	40.0	
	let-7e	40.0	40.0	40.0	40.0	31.4	40.0	
	let-7f	36.1	40.0	40.0	40.0	40.0	30.6	
	let-7a	0.0	8.5	3.8	10.6	11.2	5.2	ΔCt (mismatched - perfectly matched)
	let-7b	12.7	0.0	4.9	12.7	12.7	12.7	
	let-7c	9.9	4.5	0.0	10.2	12.9	12.9	
	let-7d	12.6	12.6	12.6	0.0	12.6	12.6	
	let-7e	8.6	8.6	8.6	8.6	0.0	8.6	
	let-7f	5.5	9.4	9.4	9.4	9.4	0.0	

Synthetic miRNA target


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let-7a  ugagguaguagguuguauaguu
let-7b  ugagguaguagguugugugguu
let-7c  ugagguaguagguuguaugguu
let-7d  agagguaguagguugcauagu
let-7e  ugagguaggagguuguauagu
let-7f  ugagguaguaauuguauaguu
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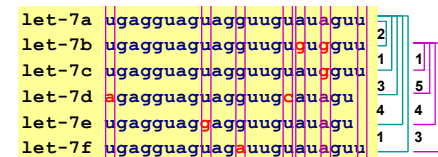
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TRADEMARKS/LICENSING