

Sensitive and specific detection of microRNAs and other noncoding RNAs



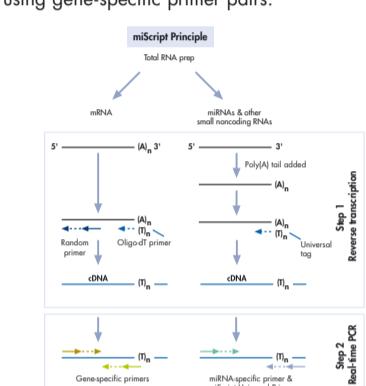
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Introduction

MicroRNAs (miRNAs) are endogenous, 21–22 nt, noncoding RNAs that mediate post transcriptional gene regulation. miRNAs are involved in regulation of gene expression during development, differentiation, cell proliferation, and apoptosis. Misregulation of miRNA expression is associated with several cancers and other diseases. We have developed the miScript System for real-time PCR analysis of hundreds of miRNAs, sno RNAs, piRNAs, other small noncoding RNAs, and also mRNAs, from a single cDNA synthesis reaction (see flowchart).

miScript principle:

- Total RNA (including small RNA) is purified from animal tissues or cells using an miRNeasy Kit.
- miRNAs and small RNAs which are not polyadenylated in nature are polyadenylated by poly(A) polymerase (miScript Reverse Transcription Kit).
- RNA is simultaneously reverse transcribed using oligo-dT primers and random primers. The oligo-dT primers carry a universal tag sequence on the 5' end (miScript Reverse Transcription Kit).
- The cDNA serves as the template for SYBR® Green based real-time PCR analysis using the miScript Universal Primer which binds to the universal tag sequence in combination with an miRNA-specific primer (miScript SYBR Green PCR Kit and miScript Primer Assay).
- The cDNA can also be used for detection of mRNA using gene-specific primer pairs.



Highly specific miRNA detection

- The existence of multiple miRNA isoforms presents a significant challenge in miRNA quantification. To determine whether the miScript System can distinguish between isoforms, primer specificity was tested. The Let-7 family was used as a model as its members have mismatches of 1 or more nucleotides or differ in length. Synthetic Let-7 isoforms were used for these experiments (Table 1).
- The results shown in Table 2 indicate that miScript Primer Assays are highly specific and can distinguish between isoforms. In most cases, cross reactivity was very low and insignificant. Where cross reactivity was observed, it was at low levels (e.g., ~6% for Let-7a miScript Primer Assay with Let-7f cDNA).

Table 2. Specificity of miScript Primer Assays for Let-7 family

cDNA used	Relative detection (as % of perfect match)							
	miRNA primer assay used							
in PCR	Let-7a	Let-7b	Let-7c	Let-7d	Let-7e	Let-7f	Let-7g	Let-7i
Let-7a	100.00	0.00	0.29	0.33	2.44	0.01	0.00	0.00
Let-7b	0.00	100.00	1.68	0.00	0.00	0.01	0.00	0.00
Let-7c	0.27	0.14	100.00	0.00	0.00	0.00	0.00	0.00
Let-7d	4.11	0.00	0.03	100.00	0.01	0.00	0.00	0.00
Let-7e	1.23	0.00	0.01	0.01	100.00	0.00	0.00	0.00
Let-7f	5.77	0.00	0.00	0.00	0.00	100.00	0.00	0.00
Let-7g	0.01	0.00	0.00	0.00	0.00	0.00	100.00	0.00
Let-7i	0.00	0.00	0.00	0.00	0.00	0.00	0.00	100.00

Table 1. Isoforms of human Let-7 family

miRNA sequence
Let-7a UGAGGUAGUAGGUUAUAGUU
Let-7b UGAGGUAGUAGGU <u>GUC</u> GUU
Let-7c UGAGGUAGUAGGU <u>AUQ</u> GUU
Let-7d <u>A</u> GAGGUAGUAGGU <u>CAU</u> GUU•
Let-7e UGAGGUAG <u>GA</u> GGGUUAUAGUU•
Let-7f UGAGGUAG <u>GU</u> GUUAUAGUU
Let-7g UGAGGUAG <u>GU</u> GUUG <u>AC</u> GUU•
Let-7i UGAGGUAG <u>GU</u> GUUG <u>GC</u> GUU•

These sequences show the Let-7 isoforms. Base changes are red and underlined. Changes in length are indicated by a red dot.

Synthetic miRNA [10⁶ copies] of each Let-7 isoform was used to generate cDNA. cDNA [10⁶ copies/reaction] was used as a template in real-time PCR analysis using a different miScript Primer Assay for each isoform. The percentage activity was determined as a proportion of the activity observed when the miScript Primer Assay was used with the cDNA of its specific isoform (i.e., perfectly matched) which was set at 100%.

miRNA profiling in Jurkat cells

- Jurkat cells were used as a model system to study expression profiling of various miRNAs. Expression of 328 different miRNAs (chosen from miRBase V 8.0; <http://microrna.sanger.ac.uk/sequences>) was analyzed using a single cDNA synthesis reaction. Of these, 111 miRNAs were detected in Jurkat cells (Figure 6).
- Changes in miRNA expression in Jurkat cells were studied in untreated cells, cells treated with Phorbol Myristyl Acetate (PMA), and cells treated with PMA and Ionomycin (CI). After 24 hours of treatment, the miScript System was used to detect changes in the miRNA expression levels (Figure 7). Studies to investigate the significance of these changes in T-cell activation are ongoing.

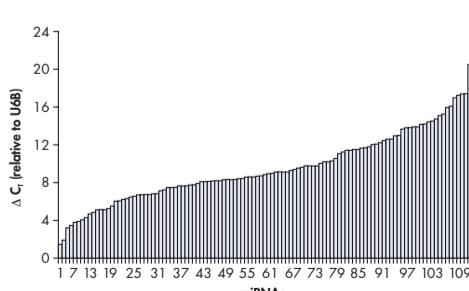


Figure 6. Expression of various miRNAs in Jurkat cells. Total RNA was prepared from untreated Jurkat cells using the miRNeasy Mini Kit. The miScript System was used with different miScript Primer Assays and 0.5 ng cDNA per reaction for real-time PCR analysis of 328 miRNAs. This graph shows the difference in C_t values between the target miRNA and the reference small nuclear RNA, U6B.

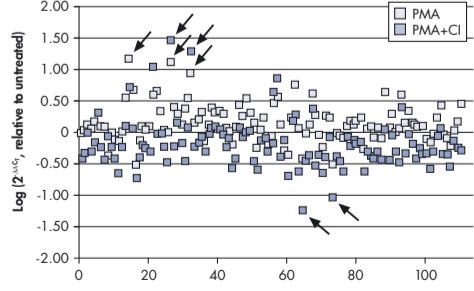


Figure 7. Changes in miRNA expression detected after different treatments. Jurkat cells were untreated, treated with PMA, or treated with PMA and CI. After 24 hours, total RNA was prepared using the miRNeasy Mini Kit. The miScript System was used with different miScript Primer Assays for real-time PCR analysis of 328 miRNAs. Data for the 111 miRNAs expressed in Jurkat cells were normalized to that of U6B and are presented here as log of 2^{ΔCt} relative to the expression in untreated cells.

High sensitivity and wide dynamic range

- Using the miScript System, cDNA was synthesized from 10 pg to 1 µg of starting RNA. This cDNA was used as a template for real-time PCR. C_t values obtained were highly linear (Figure 1). Highly linear cDNA synthesis ensures quantitative conversion of input RNA into cDNA and allows accurate quantification of miRNAs of interest.
- Synthetic miR-16 was used to generate cDNA. Amounts from 20 copies to 2 × 10⁸ copies of this cDNA were used as templates for real-time PCR using the miScript System. Detection was equally efficient for low to high template amounts (Figure 2). The wide dynamic range of the system ensures accurate reflection of miRNA expression levels.

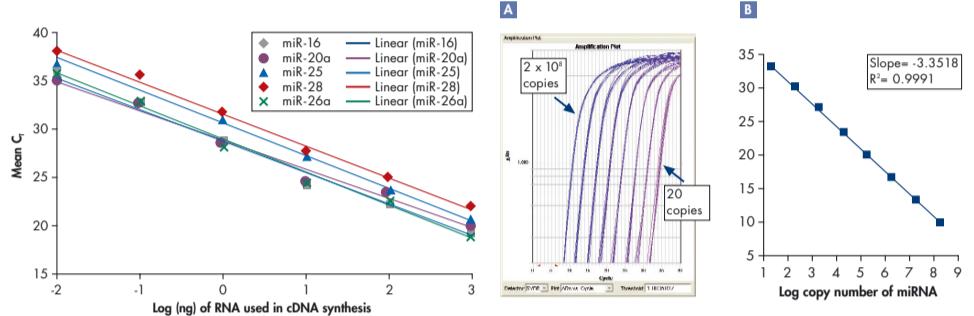


Figure 1. Highly linear cDNA synthesis reactions. RNA was purified from HeLa S3 cells using the miRNeasy Mini Kit. A range of amounts of RNA from 10 pg to 1 µg were used in cDNA synthesis reactions using the miScript Reverse Transcription Kit. cDNA was used as a template in real-time PCR assays using the miScript System. Results for 5 miRNAs are shown.

Figure 2. Accurate detection of high to low miRNA copy numbers. Synthetic miR-16 (2 × 10⁸ copies) was used to generate cDNA using the miScript Reverse Transcription Kit. A range of amounts from 20 copies to 2 × 10⁸ copies of this cDNA was used in real-time PCR using the miScript System. The resultant amplification plot and standard curve show that C_t values decreased linearly with increasing miRNA copy number indicating sensitive detection from a wide range of template amounts.

Detection of mRNA and noncoding RNAs other than miRNA

- cDNA made with the miScript System was used for detection of both miRNAs and mRNAs (Figure 3). This allows simultaneous detection of miRNAs and reference genes or other mRNAs of interest, such as an mRNA targeted by a particular miRNA.
- A range of small nucleolar RNAs (snoRNAs) and 5S ribosomal RNA were detected in HeLa S3 cells (Figure 4). Several of these snoRNAs, such as U6B, and ribosomal RNAs, such as 5S, are commonly used as reference RNAs to normalize expression levels of miRNAs.

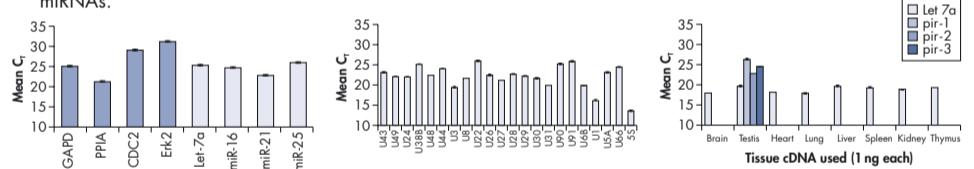


Figure 3. A single cDNA synthesis reaction enables detection of multiple miRNAs and mRNAs. Total RNA was prepared from HeLa S3 cells using the miRNeasy Mini Kit. The miScript System and 1 ng cDNA were used with miScript Primer Assays for real-time PCR analysis of 4 miRNAs (Let-7a, miR-16, miR-21, and miR-25). QuantiTec® Primer Assays were used with 1 ng cDNA and the QuantiTec SYBR Green PCR Kit for real-time PCR analysis of 4 mRNAs (GAPDH, PPIA, CDC2, and Erk2).

Figure 4. Detection of multiple snoRNAs. Total RNA was prepared from HeLa S3 cells using the miRNeasy Mini Kit. The miScript System was used with custom assays and 0.5 ng cDNA per reaction for real-time PCR analysis of 21 snoRNAs and 5S ribosomal RNA.

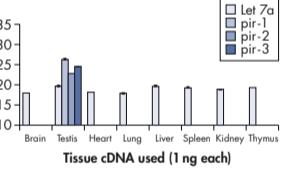


Figure 5. Tissue-specific detection of piRNAs. Total RNA was prepared from various mouse tissues using the miRNeasy Mini Kit. cDNA was prepared using 1 µg RNA from each tissue. The miScript System was used with custom assays and 1 ng cDNA/reaction for real-time PCR analysis of 3 piRNAs and the miRNA Let-7a. The piRNAs were only detected in testis tissue while Let-7a was detected in all tissues.

Summary and conclusions

- The miScript System enabled detection and quantification of multiple miRNAs from a single cDNA synthesis reaction.
- cDNA synthesis and real-time PCR analysis were highly linear over a wide range of starting RNA amounts indicating that the miScript System is highly sensitive and accurately reflects miRNA expression.
- miScript Primer Assays could distinguish between different miRNA isoforms ensuring specific detection.
- Both miRNA and mRNA could be quantified from the same cDNA synthesis reaction allowing simultaneous detection of reference genes or other mRNAs of interest.
- In addition to miRNAs, a range of snoRNAs and piRNAs were effectively quantified.
- The miScript System was used to generate a comprehensive profile of miRNA expression in untreated Jurkat cells. The system also detected changes in miRNA expression in Jurkat cells after treatment with inducing agents.
- Analysis in Jurkat cells showed that the miScript system has 3 orders of magnitude more dynamic range than microarrays. A large fraction of the miRNAs in a cell may not be detected if relying on microarray analysis alone.

miRNA resources

Useful miRNA resources are available at www.qiagen.com/miRNA including:

- Protocols and application data
- References and background information
- Links to databases

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