Expression profiling of miRNA and mRNA by real-time PCR

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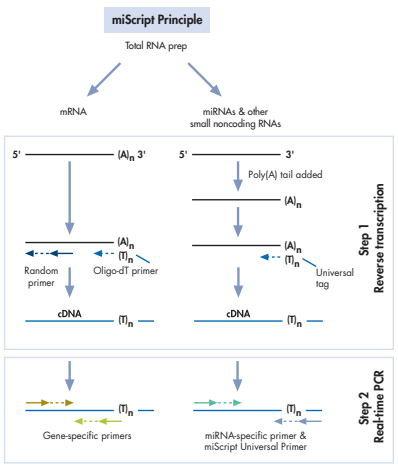
Introduction

MicroRNAs (miRNAs) are endogenous, noncoding RNAs of about 21–23 nucleotides. They 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. To help further understand the expression pattern and function of miRNAs, we developed the miScript System. When used together with QuantiTect® Primer Assays, the system can detect and quantify both miRNAs and mRNAs from the same sample.

The miScript System consists of 2 steps (see flowchart):

- **Reverse transcription**: Poly(A) tails are added to miRNAs and other nonpolyadenylated small RNAs. miRNAs and mRNAs are then reverse transcribed using a mix of oligo-dT primers and random primers. The oligo-dT primers carry a universal tag sequence.
- **Real-time PCR**: miRNAs are detected and quantified by SYBR® Green-based real-time PCR. The reaction contains an miRNA-specific primer (miScript Primer Assay) and a primer that recognizes the universal tag sequence. mRNAs are quantified by SYBR Green-based real-time PCR using gene-specific primers (QuantiTect Primer Assays).



Optimized solutions for mRNA quantification

- QuantiTect Primer Assays are bioinformatically validated, genomewide primer sets for use in real-time RT-PCR with SYBR Green detection. The assays provide high specificity and sensitivity in gene expression analysis, and are ideal for applications such as validation of RNAi or microarray data (Figure 1). Assays are available for many species, including human, mouse, and rat.
- Highly specific and sensitive results are guaranteed when QuantiTect Primer Assays are used in combination with optimized master mixes from QIAGEN. These are QuantiFast® SYBR Green Kits (for fast cycling) and QuantiTect SYBR Green Kits (for standard cycling). Both are compatible with any real-time cycler.

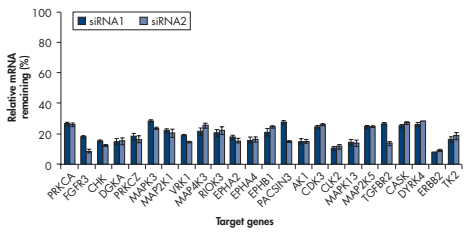


Figure 1. Reliable knockdown determination with QuantiTect Primer Assays. QuantiTect Primer Assays were used in SYBR Green-based real-time RT-PCR to assess target gene knockdown in MCF-7 cells that were transfected 48 hours earlier with HP Validated siRNAs (QIAGEN). mRNA levels were normalized and calculated relative to levels in untransfected cells (set at 100%).

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).

Table 1. Isoforms of human Let-7 family

	miRNA sequence
Let-7a	UGAGGUAGUAGGUUGUAUAGUU
Let-7b	UGAGGUAGUAGGUUGUGUGGUU
Let-7c	UGAGGUAGUAGGUUGUAUGGUU
Let-7d	AGAGGUAGUAGGUUGCAUAGU•
Let-7e	UGAGGUAGGAGGUUGUAUAGU•
Let-7f	UGAGGUAGUAGAUUGUAUAGUU
Let-7g	UGAGGUAGUAGUUUGUACAGU•
Let-7i	UGAGGUAGUAGUUUGUGCUGU•

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

- 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

Synthetic miRNA (10^{10} copies) of each Let-7 isoform was used to generate cDNA. cDNA (10^7 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%.

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 2). 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 3). 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.
- Piwi-interacting RNAs (piRNAs) are a newly reported class of germline-specific small RNAs (29-30 nt) first described in mouse testis. Three piRNAs tested were successfully detected in mouse testis tissue (Figure 4). This indicates that the miScript System will be capable of detecting any novel small RNAs discovered in the future.

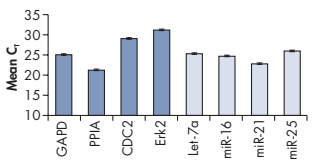


Figure 2. A single cDNA synthesis reaction enables detection of multiple miRNAs and mRNAs. Total RNA was prepared from HeLa S3 cells using the miRNeasy Mini Kit. The miScript System and 1 ng cDNA were used with miScript Primer Assays for real-time PCR analysis of 4 miRNAs (Let-7a, miR-16, miR-21, and miR-25). QuantiTect Primer Assays were used with 1 ng cDNA and the QuantiTect SYBR Green PCR Kit for real-time PCR analysis of 4 mRNAs (GAPDH, PPIA, CDC2, and Erk2).

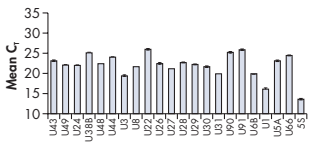


Figure 3. Detection of multiple snoRNAs. Total RNA was prepared from HeLa S3 cells using the miRNeasy Mini Kit. The miScript System was used with custom assays and 0.5 ng cDNA per reaction for real-time PCR analysis of 21 snoRNAs and 5S ribosomal RNA.

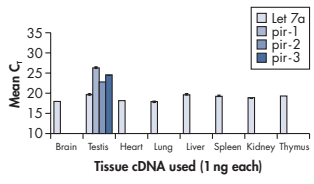


Figure 4. Tissue-specific detection of piRNAs. Total RNA was prepared from various mouse tissues using the miRNeasy Mini Kit. cDNA was prepared using 1 µg RNA from each tissue. The miScript System was used with custom assays and 1 ng cDNA/reaction for real-time PCR analysis of 3 piRNAs and the miRNA Let-7a. The piRNAs were only detected in testis tissue while Let-7a was detected in all tissues.

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 5).

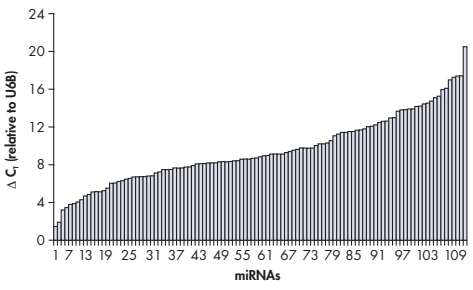


Figure 5. Expression of various miRNAs in Jurkat cells. Total RNA was prepared from untreated Jurkat cells using the miRNeasy Mini Kit. The miScript System was used with different miScript Primer Assays and 0.5 ng cDNA per reaction for real-time PCR analysis of 328 miRNAs. This graph shows the difference in C_T values between the target miRNA and the reference small nuclear RNA, U6B.

- 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 6). Studies to investigate the significance of these changes in T-cell activation are ongoing.

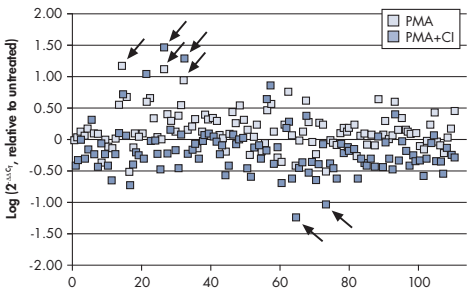


Figure 6. Changes in miRNA expression detected after different treatments. Jurkat cells were untreated, treated with PMA, or treated with PMA and CI. After 24 hours, total RNA was prepared using the miRNeasy Mini Kit. The miScript System was used with different miScript Primer Assays for real-time PCR 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 $2^{-\Delta\Delta CT}$ relative to the expression in untreated cells.

Summary and conclusions

- The miScript System enables detection and quantification of multiple miRNAs from a single cDNA synthesis reaction.
- miScript Primer Assays can distinguish between different miRNA isoforms, allowing specific detection.
- The miScript System detects miRNAs by SYBR Green-based real-time PCR and is fully compatible with QuantiTect Primer Assays for quantification of mRNA transcripts.
- The unique combination of miScript Primer Assays and QuantiTect Primer Assays enables quantification of both miRNAs and mRNAs from the same cDNA synthesis reaction.
- 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

GeneGlobe™ Web portal

miScript Primer Assays and QuantiTect Primer Assays are easily ordered online at www.qiagen.com/GeneGlobe . This comprehensive Web portal also offers a wide range of other gene-specific products, such as genomewide siRNAs, validated siRNAs, and protein assays.

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