Introduction to an advanced microRNA technology

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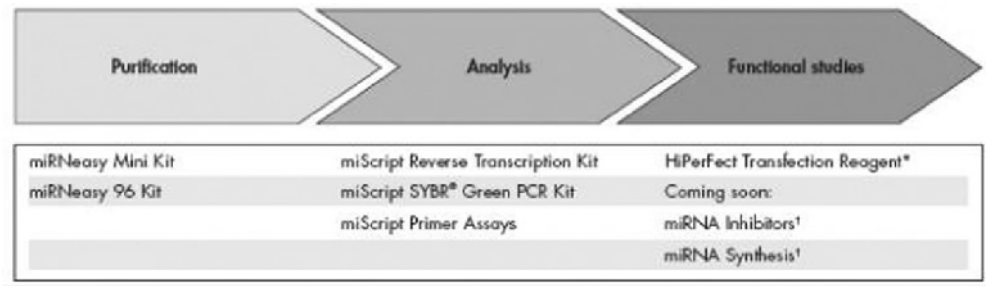
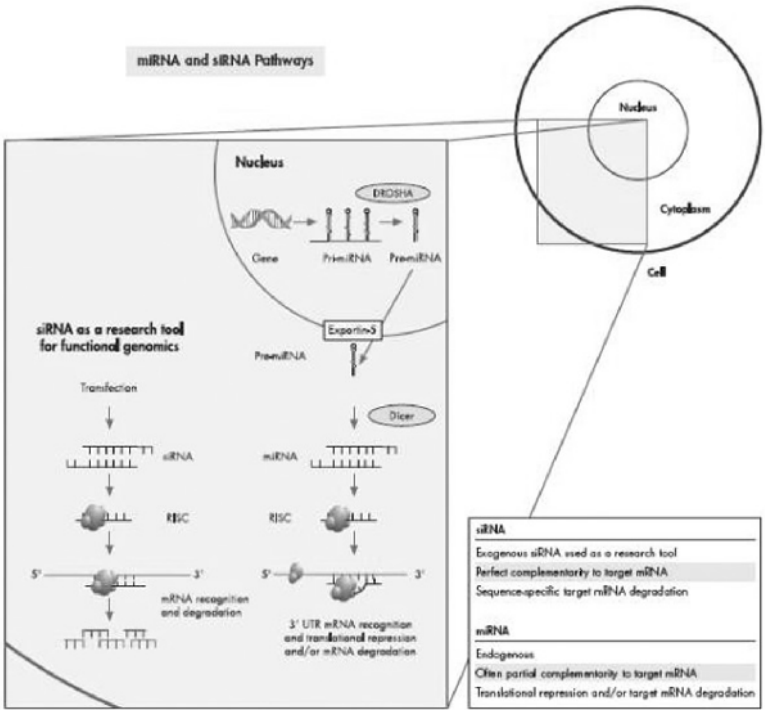
In the last few years, the identification of microRNA (miRNA) and the recognition of its important role in regulation of gene expression have led to increasing interest in the identification and characterization of miRNAs. A growing body of evidence suggests that miRNAs play a role in many diverse biological processes such as development, differentiation, and apoptosis. Misregulation of miRNA expression is reported to be associated with several cancers and other diseases. The miRNA system is an endogenous mechanism of regulation of gene expression. miRNA precursors are transcribed from genomic DNA in the nucleus. The mature miRNAs contribute to the regulation of endogenous genes, primarily by translational repression (see flowchart, opposite). In addition, miRNAs can mediate mRNA destruction by rapid deadenylation and/or decapping. While siRNAs and miRNAs act on endogenous mRNAs through integration into a multiprotein silencing complex (RISC), siRNAs require a perfect match with the targeted mRNA, leading to cleavage and subsequent destruction, whereas miRNAs are often only partially complementary to target mRNAs. Naturally occurring miRNA-binding sites are typically found in the 3' untranslated regions (UTRs) of target mRNAs. Their partial complementarity has made positive identification of true binding sites difficult and imprecise.

To support this exciting new field of research, QIAGEN has introduced innovative miRNeasy and miScript technologies for miRNA purification and detection. miRNeasy Kits enable purification of total RNA, including miRNAs and other small RNAs, from all types of animal tissues and cells. Small RNAs, including miRNA can be enriched in a separate fraction if desired. Using the miScript System, real-time PCR analysis can be used to detect hundreds of miRNAs, as well as mRNAs, from a single cDNA synthesis reaction. As well as miRNAs, knowledge about other classes of small, noncoding RNAs is currently emerging. miRNeasy Kits and the miScript System have been tested and verified for purification and detection of other noncoding RNAs (e.g., snoRNAs, piRNAs).

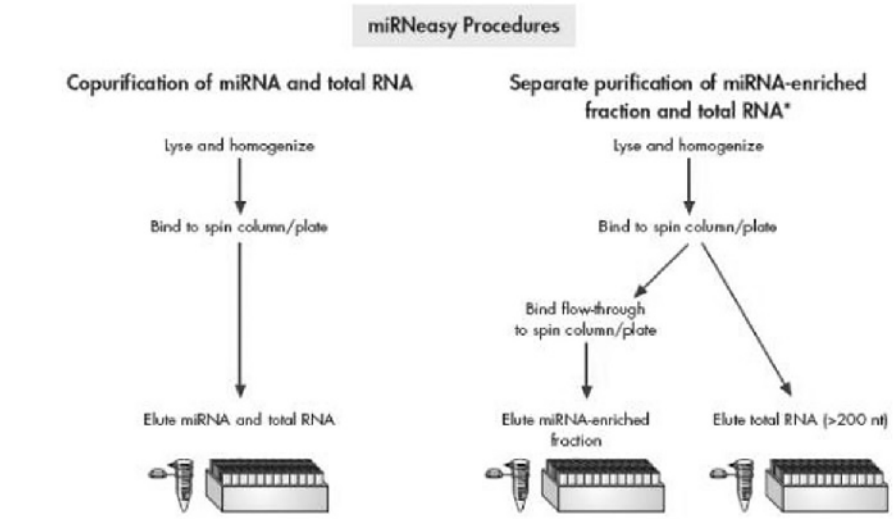


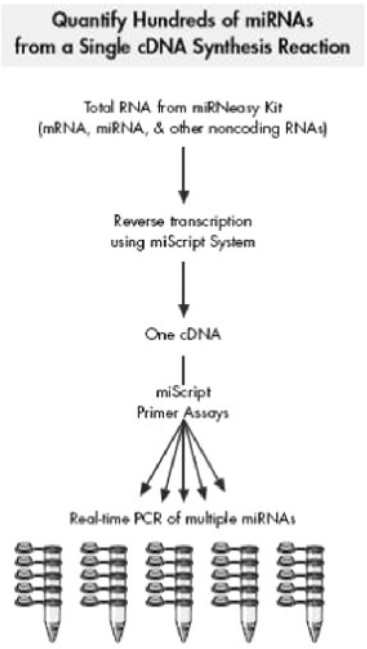


miRNAs are first transcribed in the nucleus as long, primary miRNAs (pri-miRNA). While still in the nucleus, they are processed into precursor miRNAs (pre-miRNA) by nuclear Drosha, a dsRNA-specific ribonuclease. Pre-miRNAs are subsequently transported from the nucleus into the cytosol by Exportin-5. Dicer (an RNase III-like enzyme) processes the pre-miRNAs into ~22 nt mature miRNAs that are subsequently incorporated into the RNA-induced silencing When the RISC identifies the complementary or partially complementary mRNA target, it inhibits gene expression by translational repression or by mRNA degradation.

1. Purification of total RNA or enrichment of miRNA

miRNeasy Kits enable purification of total RNA which includes RNA from approximately 18 nucleotides (nt) upwards. Alternatively, an miRNA-enriched fraction (<200 nt) and a total RNA fraction (>200 nt) can be purified separately (see flowchart). The miRNeasy Mini Kit provides low-throughput RNA purification using spin columns. The miRNeasy 96 Kit is used for highthroughput purification in a 96-well format. Purified total RNA is suitable for use in downstream applications such as northern blot analysis and quantitative, real-time RT-PCR. Enrichment of small RNAs may provide better results for some applications such as microarray analysis.





2. cDNA synthesis reaction using SYBR Green based, real-time PCR

Principle of the miScript System

The miScript System is a three-component system which covers all the steps of conversion of miRNA and mRNA into cDNA and detection of miRNAs in SYBR Green based, real-time PCR. The miScript Reverse Transcription Kit, miScript SYBR Green PCR Kit, and miScript Primer Assay allow sensitive and specific detection and quantification of miRNA. The modular system enables detection of individual miRNAs of interest using miScript Primer Assays or screening of multiple human, mouse, or rat miRNAs using miScript Primer Assay Sets. Alternatively, researcher-designed assays can be used for newly discovered miRNAs. For this reason, the three components can be purchased separately.

The miScript System comprises the following components:

- miScript Reverse Transcription Kit
  This kit enables simple, single-step cDNA synthesis.
- miScript SYBR Green PCR Kit
  This kit includes the miScript Universal Primer which allows detection of miRNAs in

combination with an miRNA-specific primer.

- miScript Primer Assay
  The assay comprises an miRNA-specific forward primer which is used in combination with the miScript SYBR Green PCR Kit.

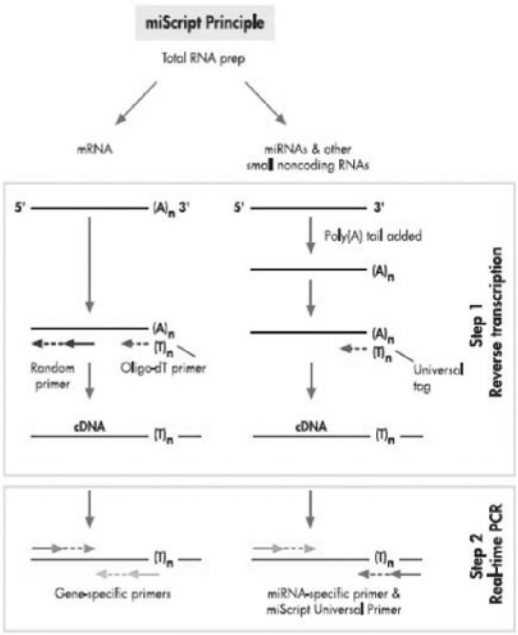
miScript Reverse Transcription Kit

The miScript Reverse Transcription Kit includes miScript Reverse Transcriptase Mix and miScript RT Buffer. miScript Reverse Transcriptase Mix is an optimized blend of enzymes comprising a poly(A) polymerase and a reverse transcriptase. miScript RT Buffer has been developed specifically for use with miScript Reverse Transcriptase Mix to enable maximum activity of both enzymes. It includes Mg2+, dNTPs, oligo-dT primers, and random primers.

Unlike mRNAs, miRNAs are not polyadenylated in nature. During the reverse-transcription step, miRNAs are polyadenylated by poly(A) polymerase. In parallel, reverse transcriptase converts both miRNA and mRNA to cDNA . The parallel reactions make miRNA conversion into cDNA a very fast and simple one-tube procedure. The oligo-dT primers carry a universal tag sequence on the 5' end which allows amplification in the real-time PCR step.

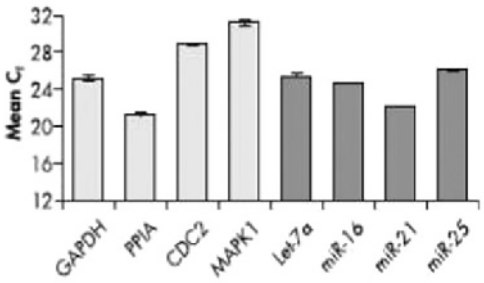
miScript SYBR Green PCR Kit

The miScript SYBR Green PCR Kit is based on the proven technology of QuantiTect® SYBR Green Kits. The miScript SYBR Green PCR Kit includes QuantiTect SYBR Green PCR Master Mix and the miScript Universal Primer which is specific for the universal tag sequence on the 5' end of the cDNA. The cDNA serves as the template for real-time PCR analysis using the

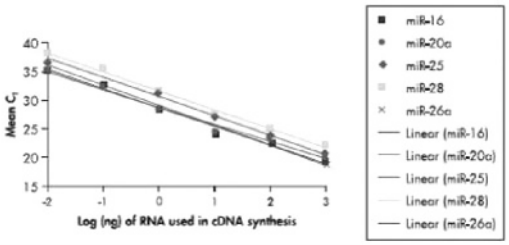


miScript Primer Assay in combination with the miScript SYBR Green PCR Kit. miRNAs are amplified using the miScript Universal Primer together with the miRNA-specific primer (the miScript Primer Assay).

A single cDNA synthesis reaction is sufficient to interrogate hundreds of miRNAs by real-time PCR using different miScript Primer Assays, allowing comprehensive expression profiling of known miRNAs. The cDNA can also be used for detection of mRNA using gene-specific primer pairs (instead of the miScript Primer Assay and Universal Primer), allowing simultaneous detection of reference genes, such as GAPDH, or any other mRNAs of interest, such as the mRNA targeted by a particular miRNA . QIAGEN offers QuantiTect Primer Assays for real-time PCR detection of mRNA.



[Figure 1] 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 was used for real-time PCR analysis of 4 miRNAs (Let-7a, miR-16, miR-21, and miR-25). QuantiTect Primer Assays were used for real-time PCR analysis of 4 mRNAs (GAPDH, PPIA, CDC2, and MAPK1).



[Figure 2] 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 1μg were used in cDNA synthesis reactions using the miScript Reverse Transcription Kit. cDNA was used as a template in quantitative, real-time PCR assays for 5 miRNAs (miR-16, miR-20a, miR-25, miR-28, and miR-26a).

miScript Primer Assay

miScript Primer Assays are miRNA-specific primers used in combination with the miScript SYBR Green PCR Kit for real-time PCR of miRNA using SYBR Green detection. The miScript SYBR Green PCR Kit includes the miScript Universal Primer which is necessary for successful amplification. miScript Primer Assays are available in single tubes and can be ordered online from the GeneGlobe Web portal (www.qiagen.com/GeneGlobe). miScript Primer Assays for the detection of human miRNAs have been experimentally validated. miScript Primer Assays for mouse and rat are predesigned and a proportion has been validated. Human, Mouse, or Rat miScript Primer Assay Sets provide assays for >95% of the miRNAs listed in miRBase version 9.0 (http://microrna.sanger.ac.uk/sequences/).

Linearity of cDNA synthesis enables sensitive detection

Using the miScript System, the cDNA synthesis reaction is highly linear which ensures sensitive, accurate quantification in subsequent real-time PCR. In the results shown in Figure 6, CT values from real-time PCRs were highly linear following cDNA synthesis reactions from a range of amounts of starting RNA.

Highly specific miRNA detection

The existence of multiple miRNA isoforms presents a significant challenge in miRNA quantification. miScript Primer Assays are highly specific and can distinguish between isoforms as shown for the Let-7 family. Human Let-7 isoforms with mismatches and/or differing lengths were used in these experiments. 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). These results indicate that miScript Primer Assays are isoform specific.

[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.

[Table 2] Specificity of miScript Primer Assay

	Relative detection (as % of perfect match)							
cDNA used in PCR	miScript Primer Assay used							
	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 miRNAs of each Let-7 isoform were used in cDNA synthesis reactions performed with the miScript Reverse Transcription Kit. An aliquot of the resultant cDNA was used as a template in real-time PCR reactions with a miScript Primer Assay for each isoform and the miScript SYBR Green PCR Kit. The % relative detection was calculated using the differences between the CT values achieved from the mismatching miScript Primer Assays and those from the perfectly matching miScript Primer Assays (% relative detection = 2– _CT x 100).