Scientific article

# miRNA biomarker discovery — overcoming limiting sample material

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**Abstract**: microRNAs (miRNAs) exhibit a tightly regulated spatial and temporal pattern of expression during development and differentiation. Furthermore, miRNAs have been shown to be aberrantly expressed in cancer and other diseases, and may prove to be excellent diagnostic, theranostic, and prognostic biomarkers. Often the most valuable and informative samples — such as laser-captured samples, circulating tumor cells, or extracellular miRNA in body fluids — are the hardest to obtain in amounts sufficient for detailed miRNome profiling. We present an integrated, PCR-based system that reduces the amount of sample required for full miRNome profiling by several orders of magnitude and provides unparalleled reproducibility and precision. This advance enables detailed miRNA analysis on the smallest of samples and opens up new possibilities for biomarker development.

## Introduction

The discovery of the first miRNA in 1993 (1) provided only a hint of the extent to which miRNA function is intertwined in virtually every process in mammalian cells. Once it was discovered that miRNAs are widespread in both plant and animal kingdoms and exhibit a complex pattern of expression (2–4), the rise of the field to scientific prominence seemed inexorable. miRNAs have been shown to play a critical role in cell fate determination and in eliciting and maintaining a pre- or post-differentiated state. Most miRNA genes are transcribed by RNA Polymerase II and have regulatory elements as complex as those that regulate protein-encoding genes (5–7). In fact, many miRNA precursors are embedded in introns of protein-encoding genes and are spliced out during mRNA processing as pre-miRNA (5, 8–10). As we now know that miRNA regulates thousands of genes, it is not surprising that Croce and others have discovered that many miRNAs are deregulated in cancer and other diseases, and that this altered expression can be used to identify and classify subtypes of disease (11). Even more interesting, as well as occurring in disease, miRNA deregulation has also been identified in some instances as critical to the progression of the cell from a normal to diseased state. miRNA research has made a significant impact on all aspects of biomedical research, from providing a better understanding of pathway regulation in model systems to explaining coordinated gene expression changes in cancer and creating new possibilities for molecular diagnostics and nucleic-acid–based drugs.

## Contents

## Circulating, cell-free miRNA

The remarkable discovery that stable miRNAs could be found in serum and plasma was soon confirmed and extended by many researchers (12, 13). A high level of interest, and hundreds of research papers, focused on the possibility that changes in abundance of circulating miRNAs could be adopted as noninvasive biomarkers for a variety of indications. Circulating miRNA is most certainly not naked miRNA, which would be degraded within seconds due to high levels of nucleases in blood. Several reports have demonstrated that circulating miRNA derives its stability through several mechanisms. Serum stability can result from the formation of complexes between circulating miRNA and specific proteins, such as Ago2 (14–16). Other studies have found miRNA contained within circulating exosomes and other microvesicles (17). Fractionation experiments show that both exosomal and non-exosomal miRNA make up the total extracellular miRNA repertoire in serum and other body fluids, although the composition can vary between different body fluids. While there is no longer any doubt that a stable population of extracellular miRNAs exist in circulation, nothing is positively known about their natural function. However, there are tantalizing hints that circulating miRNAs play a signaling role in both normal physiology and in promoting metastasis in cancer (18, 19). Certain types of cancer cells shed high numbers of exosomes into circulation. In fact, it has been reported that some cancer cells can selectively shed specific miRNAs to lower their intracellular concentration. Clearly there are many more exciting discoveries to be made in miRNA function and regulation.

## miRNA profiling techniques and their RNA requirements

Profiling the miRNome of a sample can be accomplished in several ways, each of which has advantages and limitations. Today, the major approaches are hybridization arrays, RNA-seq, and qRT-PCR. Screening with hybridization arrays requires relatively large microgram amounts of RNA and is limited in both sensitivity and dynamic range. The range of miRNA expression in a typical cell is several orders of magnitude larger than the dynamic range of a hybridization array, therefore a large number of expressed sequences will be undetectable. Obviously too, it is only possible to detect miRNA species that have corresponding assays on the array, meaning that this is not a suitable tool for discovery of new miRNA species.

Next-generation sequencing (NGS) of RNA, often called RNA-seq, is a relatively new technology that takes advantage of massively parallel sequencing on a solid support. This technique is highly suited to discovery, enabling characterization of SNPs, mutations, processing variants, and novel miRNA species. RNA-seq is generally regarded as a screening technology. In its current form, RNA-seq characterizes all sequences in a sample in a semi-quantitative, but exhaustively thorough, manner.

For miRNA profiling, qRT-PCR remains the preferred method, owing to its combination of low sample amount requirements, sensitivity, selectivity, and dynamic range. In a single run, miRNA targets ranging from 10 to $10^7$ copies can be accurately quantified. Sample requirements for qRT-PCR are much lower than for RNA-seq or arrays. Without any preamplification, excellent sensitivity can be achieved with less than 1 ng total RNA per assay, or approximately 1 µg RNA per 1800 assay miRNome. Furthermore, using preamplification as described in this paper, full miRNome screening can be performed, in technical triplicate, with just 10 ng total RNA. From serum or plasma, this corresponds to the miRNA content of approximately 1 µl sample. Quantitative preamplification of the miRNome is an enabling development, eliminating the requirement for microgram quantities of RNA per sample to profile the miRNome. In addition, preamplification makes profiling from other body fluids that have far less miRNA than serum, such as cerebrospinal fluid, saliva, and urine, possible without having to process an excessively large sample volume to attain sufficient RNA.

## Principle of the miScript® PCR System for qRT-PCR

While the small size of miRNAs confers some technical advantages (e.g., miRNAs are less likely than mRNA to be affected by cross-linking in FFPE samples), their 21–23 bases leave little room for maneuvering of primer or probe design to optimize an assay. While several approaches have been commercially developed, the differences between them are primarily about flexibility, as a properly designed assay using any of the currently available systems will have roughly equivalent sensitivity and selectivity.

The miScript PCR System is the only technology that includes a truly universal, small-RNA-specific cDNA synthesis reaction. In this patent-pending technology, miRNA is tailed by *E. coli*

poly A polymerase, followed by an anchored, oligo dT primed cDNA synthesis reaction. This ensures that any and all miRNAs are converted into cDNA without bias. Critically, this includes miRNAs that have not yet been characterized, ensuring that a researcher can always return to an archived cDNA sample when new miRNA targets are identified. The cDNA is tagged with a unique sequence present in the anchored primer and this serves as a common 3' PCR priming site (Figure 1).

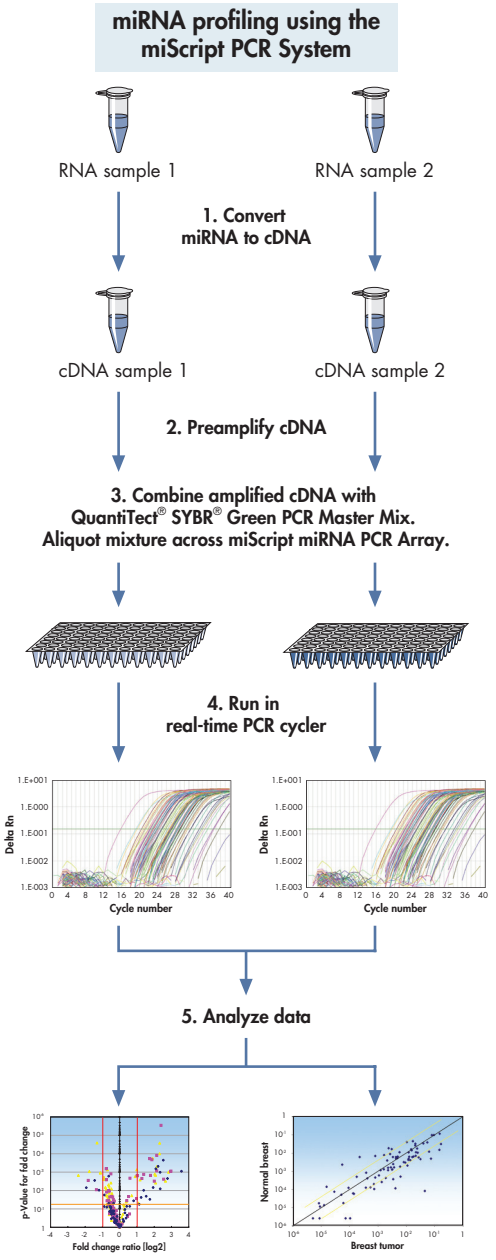


**Figure 1. miRNA expression profiling from samples containing low RNA amounts.**

miScript Primer Assays are designed with several features that favor robust, large-scale miRNA profiling. First, assays are designed with a very restricted amplicon size and $T_m$ range. This ensures uniformity of assay performance under a universal set of PCR conditions and makes melt curve analysis easy to interpret. Second, the system is designed to tolerate the 3' heterogeneity commonly found in miRNAs. Extensive NGS of miRNA has shown high levels of polymorphism at the 3' end of many different miRNAs. This data, available at miRBase (www.miRBase.org), shows that in many cases, a major fraction of a specific miRNA in a cell can have several additional or missing bases on the 3' end, resulting in mismatches to the canonical sequence (Figure 2). This does not present a problem for the reverse transcription reaction in the miScript PCR System, which will convert any miRNA into cDNA, nor does it generally present a problem for miScript Primer Assays, as they are deliberately designed so that their 3' ends do not extend to the 3' end of the mature miRNA sequence. However, as described, the alternative technology of miRNA-specific priming found in some commercially available PCR profiling technologies, would be unable to prime cDNA synthesis from these variants, resulting in an underestimation of the true levels of many miRNA species in a cell (20).

## Preamplification using the miScript PCR System

Approximately 0.5–1 ng total RNA per assay is recommended for maximum sensitivity in qRT-PCR of miRNA using the miScript PCR System. This allows quantification of as few as 10 copies of an miRNA from approximately 100–200 cells, or far less than one copy per cell (assuming 30 pg total RNA per cell). This level of sensitivity and dynamic range (approximately $10^7$) is sufficient for most experiments. However, when samples are exceptionally precious, need to be used for multiple analytes, or are simply available in very limited amounts as commonly found with isolated, circulating tumor cells, microdissected samples, or extracellular miRNA in biofluids, a preamplification reaction can enable experiments that would be otherwise impossible. Preamplification with the miScript PCR System uses just 1 µl of the 10 µl cDNA synthesis reaction as input. The volume of cDNA synthesis reaction used is critical, as RT components ▶

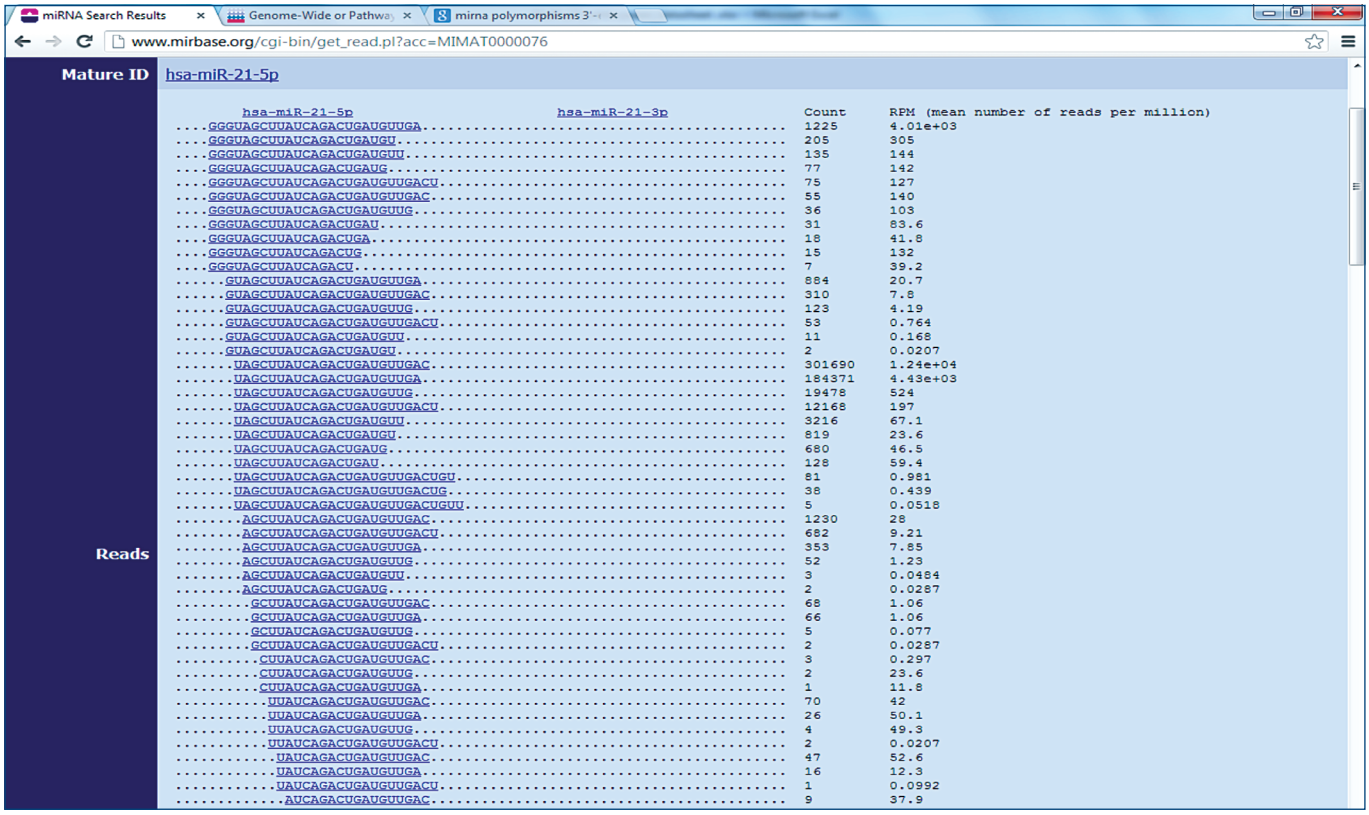


**Figure 2. Deep sequencing reads for hsa-miR-21-5p at miRBase show variant miRNA ends.** These data, available at miRBase (www.miRBase.org), show that a large fraction of a specific miRNA in a cell can have additional or missing bases at the 3' end, resulting in mismatches to the canonical sequence.

must be carried over to the preamplification reaction. The amount of RNA is less critical, but we typically recommend a cDNA synthesis reaction containing 10 ng RNA (equivalent to approximately 300 cells). Each cDNA synthesis reaction can then be used for 10 preamplification reactions, each with 1 µl input (equivalent to 1 ng RNA or 30 cells). More RNA may be used in the cDNA synthesis reaction, but it is not necessary. Less RNA may also be used, however the sampling variation from less than 3 cells equivalent of RNA increases the variability of results for moderately to rarely expressed miRNAs (Figure 3).

Preamplification is a highly optimized, highly multiplexed PCR containing either 96 or 384 assays. These assay pools correspond to QIAGEN's predeveloped 96- and 384-assay miScript miRNA PCR Arrays. For miRNomes that are larger than one 384-well plate, such as the human and mouse miRNomes, two or three 384-plex preamplification reactions must be performed. After 12 cycles of preamplification, the amplified product is mixed with real-time master mix and used for miRNA profiling. Targets as rare as 10–20 copies to targets as abundant as $10^7$ copies are preamplified by 4 orders of magnitude (Figure 4).

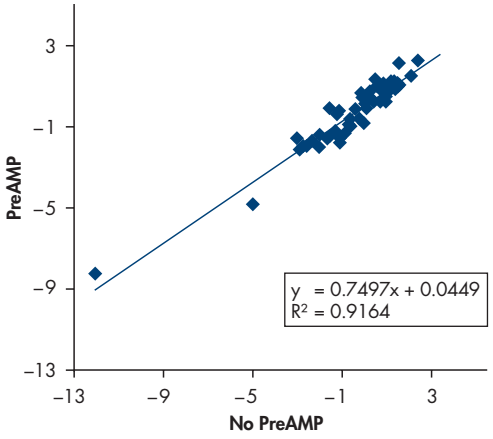


**Figure 3. Low template cDNA input increases variability of results.** Preamplified and nonpreamplified cDNA from the same preps (equivalent to less than 4 cells) and cDNA synthesis reactions were used for miRNA profiling. A scatter plot of $\Delta\Delta C_T$ values between normal lung and tumor lung FFPE tissue sections demonstrates low correlation between nonpreamplified and preamplified samples. The miRNeasy FFPE kit was used to purify RNA from normal and tumor lung tissue 5 µm FFPE sections. Reverse transcription was performed using the miScript II RT Kit with miScript HiSpec Buffer and 100 pg cDNA was used for preamplification with the miScript PreAMP PCR Kit. A 96-plex miFinder miScript PreAMP Pathway Primer Mix and Array were used for miRNA profiling.

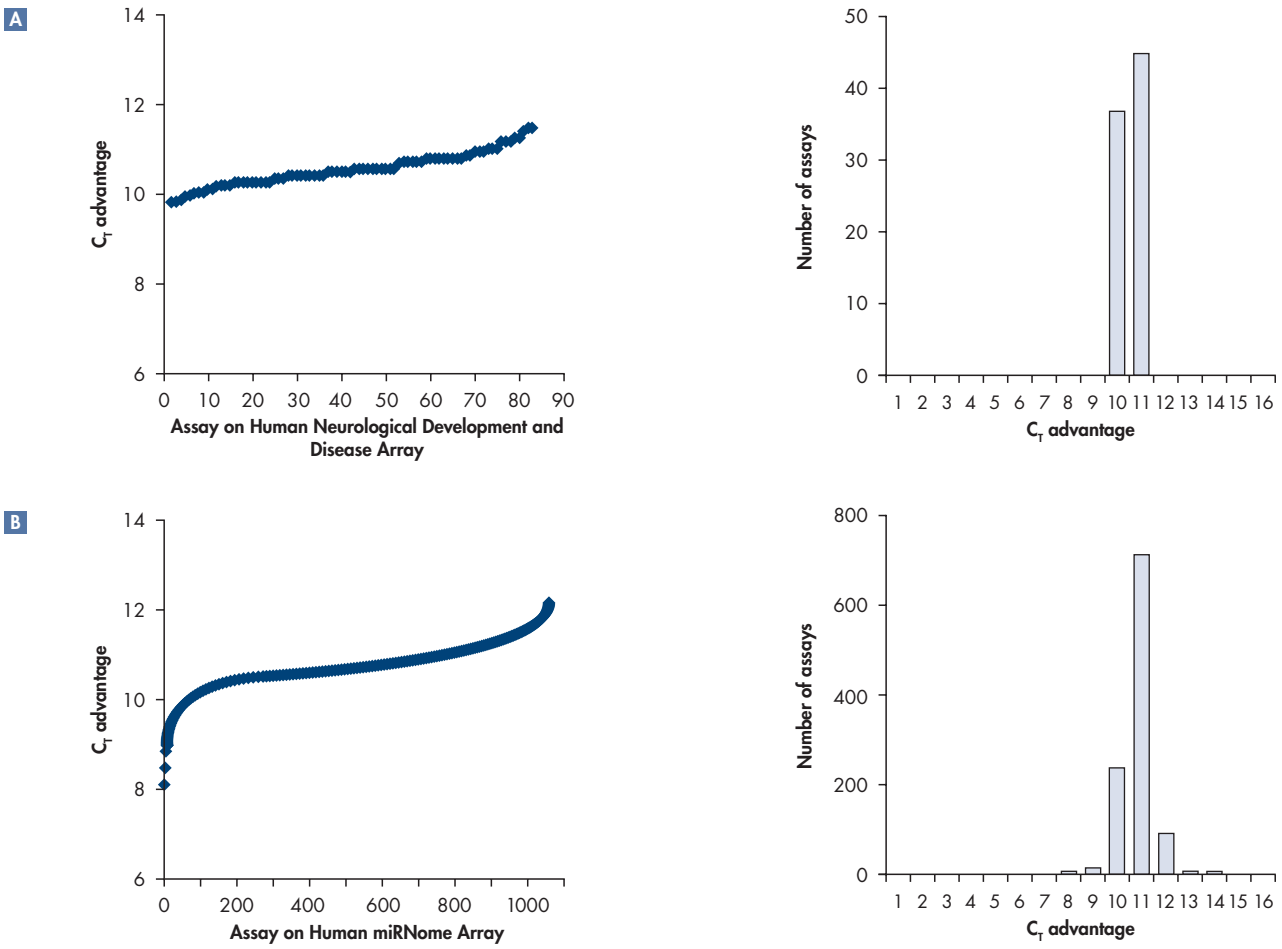


**Figure 4. $C_T$ advantage after preamplification using 96 and 384 assays.** After 12 preamplification cycles, most samples consistently show a $C_T$ advantage of between 10 and 12 cycles. These $C_T$ differences are highly reproducible. Reverse transcription was performed using the miScript II RT Kit with miScript HiSpec Buffer and a pool of synthetic miRNAs. cDNA was used for preamplification with the miScript PreAMP PCR Kit. **A** A 96-plex Human Neurological Development and Disease miScript PreAMP Pathway Primer Mix and Array or a **B** 384-plex Human miRnome miScript PreAMP Pathway Primer Mix and Array were used for miRNA profiling.

## Preservation of miRNA expression profile after preamplification

For the development of miRNA expression signatures as biomarkers using preamplified material, it is important that the preamplification reaction is extremely reproducible to preserve relative changes in miRNA expression. It is not absolutely critical that every assay has exactly the same efficiency in preamplification, although we make every effort to achieve that, but it is more important that each assay performs the same way on every sample. This is the case for optimized miScript PreAMP Primer Mixes. It is challenging to multiplex such high numbers of assays; however, several design features make this possible. First, since the miScript PCR System uses a universal 3' PCR primer, the complexity of the preamplification primer pool is reduced by 50%. Second, as mentioned earlier, all the amplicons are both the same size and very close in $T_m$, which greatly facilitates non-biased amplification in a 384-assay multiplex reaction. Finally, the chemistry of the cDNA synthesis reaction severely restricts any cDNA side reactions, so there is much less background cDNA synthesized and therefore lower background in the preamplification reaction. The robustness of the system is demonstrated in Figure 5, in which the fold-difference of expression is compared between 2 samples — one preamplified and one nonpreamplified sample. It is important to note the values on the axes are not $C_T$ or log change values, they are fold-change values. These data show that the fold changes between these 2 samples are essentially identical, despite the fact that 1000-fold less starting material was used in the preamplified sample.

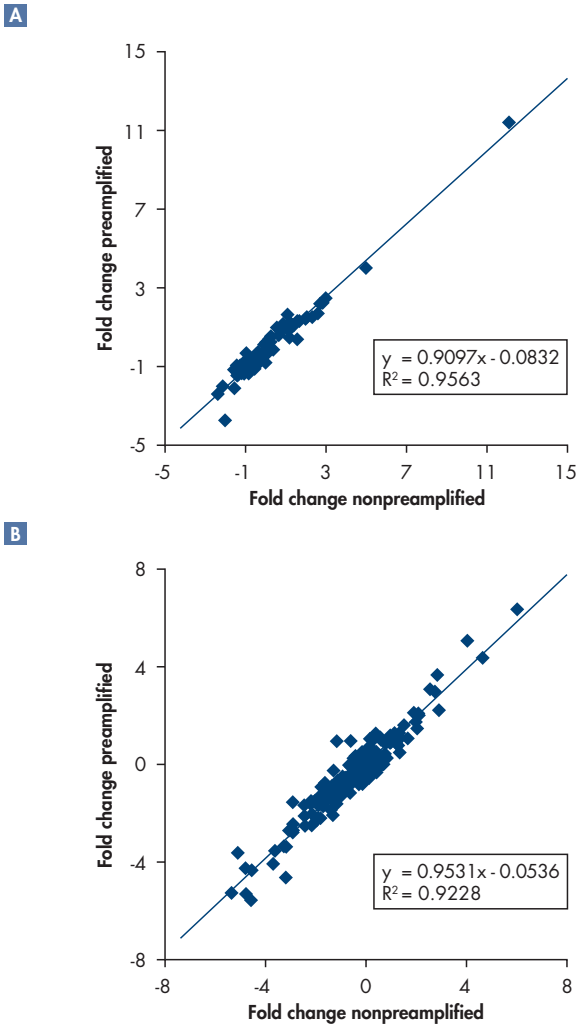


**Figure 5. Preamplification preserves expression patterns in FFPE samples using 1000-fold less input cDNA.** Preamplified cDNA and nonpreamplified cDNA from the same prep were used for miRNA profiling. Scatter plots of x-fold expression change calculations ($2^{-\Delta\Delta C_T}$) between normal and tumor sections demonstrate high correlation between nonpreamplified and preamplified samples. Normalization was performed against housekeeping controls. A 96-plex miFinder miScript PreAMP Pathway Primer Mix and Array or B 384-plex miScript PreAMP miRNome Primer Mix and Array were used. The miRNeasy FFPE Kit was used to purify RNA from normal and tumor lung tissue 5 µm FFPE sections. cDNA was prepared from 10 ng total RNA using the miScript II RT Kit with miScript HiSpec Buffer and preamplified using the miScript PreAMP PCR Kit.

## Preamplification strategies for archival, FFPE tumor samples

QIAGEN provides the miRNeasy FFPE Kit for purification of miRNA from formalin-fixed, paraffin-embedded (FFPE) tissue samples. After purification with the miRNeasy FFPE Kit, a typical 5 µm section often yields enough RNA for a miRNome profile. However, for fine needle biopsies, smaller samples, or valuable samples, far less RNA can be used to obtain high-quality miRNA expression data. In our experience, profiling data derived from adjacent sections can be extremely similar as long as the cell type and numbers are similar (Figure 6). However, due to the variations in fixation and storage, careful normalization is required. This can be accomplished by normalizing against invariant miRNA or snoRNA or by normalization against the mean of expressed miRNA targets.

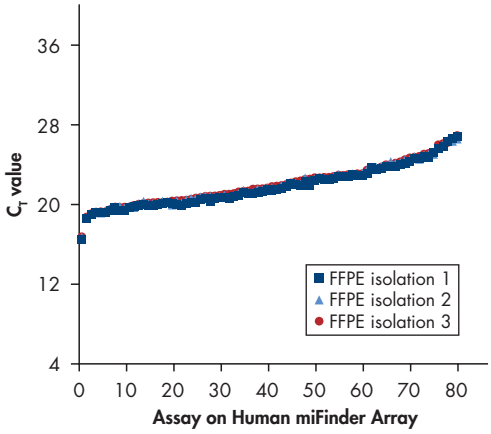


**Figure 6. Adjacent FFPE sections provide consistent expression patterns.** cDNA derived from 3 different FFPE sections from the same sample were used for miRNA profiling. $C_T$ values were highly consistent for all assays tested between the samples. The miRNeasy FFPE Kit was used to purify RNA from lung tumor FFPE samples. cDNA was prepared from 125 ng total RNA using the miScript II RT Kit with miScript HiSpec Buffer. Samples were not preamplified. The Human miFinder miScript miRNA PCR Array was used for miRNA profiling.

## Preamplification strategies for body fluids

The discovery of reproducible changes in cell-free miRNA levels in the circulation of people with various diseases has sparked great interest in developing miRNA profiles from human body fluids as biomarkers. Serum and plasma in particular have been the subject of intensive profiling. Generally, there is sufficient miRNA in just 20 µl serum or plasma for a sensitive miRNA profiling experiment by qRT-PCR. If the volume of plasma or serum is not limited, we recommend using 100–200 µl per RNA preparation. However, when samples are very valuable or even more limited, a preamplification reaction can be used to further decrease the amount of serum or plasma required. For example, following preamplification with the miScript PCR System, a full human miRNome panel can be screened in triplicate using only ~1 µl serum equivalents. In addition to the lower sample requirement, preamplification results in a significant increase in detected miRNAs (Figures 7 and 8).

Compared to plasma or serum, whole blood contains large amounts of miRNA. However, if the sample is severely limiting, for example a 1 µl blood spot, preamplification can be performed in the same way as for serum or plasma with good success.

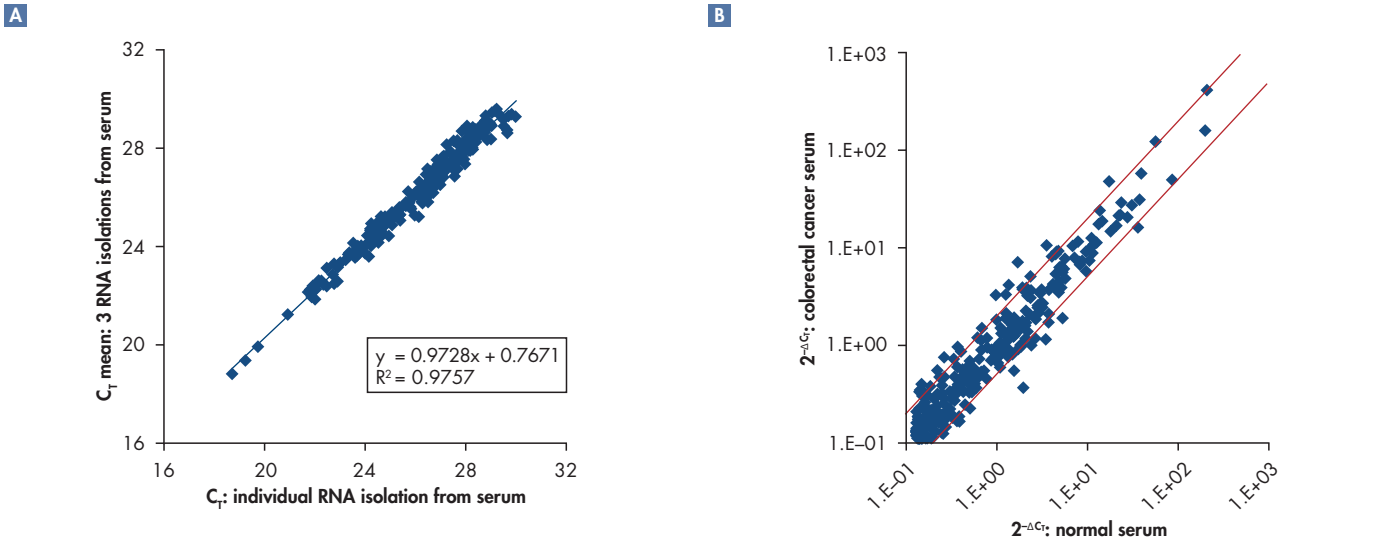


**Figure 7. Reliable miRNome profiling from <1 µl serum.** Total RNA was purified from 3 different 5 µl normal serum samples and 3 different 5 µl colorectal cancer serum samples using the miRNeasy Serum/Plasma Kit. cDNA was then prepared from 0.7 µl serum equivalents (SE) using the miScript II RT Kit with miScript HiSpec Buffer. cDNA was preamplified using the miScript PreAMP PCR Kit with Serum & Plasma 384HC miScript PreAMP Pathway Primer Mix prior to profiling. miRNA profiling was performed with the with the Serum & Plasma 384HC miScript miRNA PCR Array. Scatter plots show **A** high correlation in mean $C_T$ values achieved between 3 RNA isolations from serum and an individual RNA isolation from serum, and **B** differences in miRNA expression between normal and colorectal cancer samples.

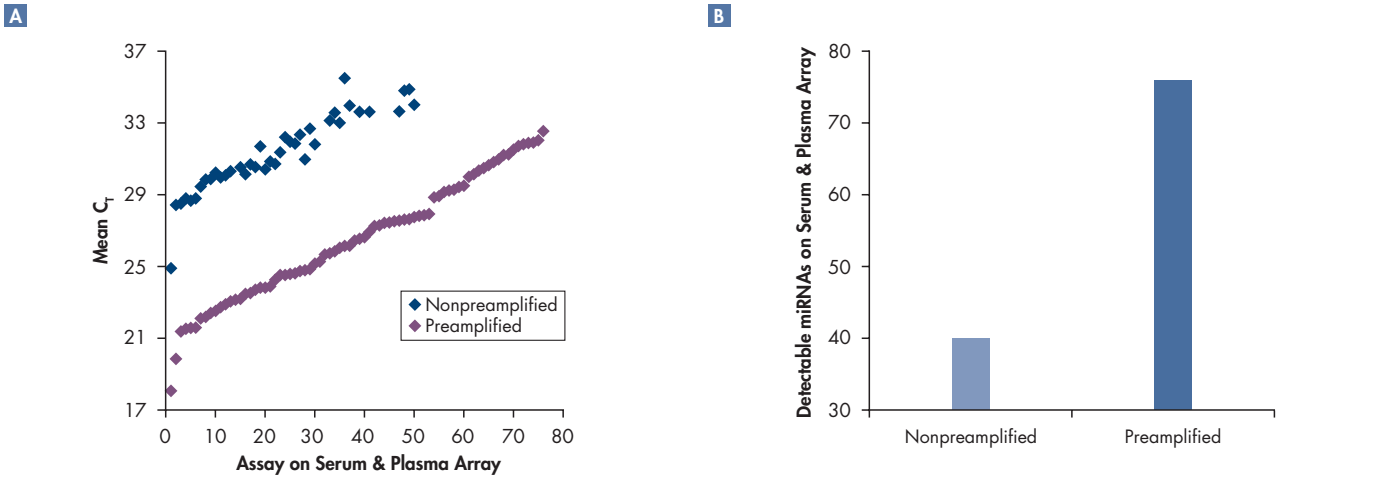


**Figure 8. 100% increase in miRNAs detected from 10-fold less cDNA after preamplification from serum.** Total RNA was purified from 5 µl human serum using the miRNeasy Serum/Plasma Kit. cDNA was then prepared from 0.7 µl serum equivalents (SE) using the miScript II RT Kit with miScript HiSpec Buffer. cDNA (0.7 µl SE) was used directly for miRNA profiling or one-tenth of the cDNA preparation (0.07 µl SE) was preamplified using the miScript PreAMP PCR Kit with Serum & Plasma miScript PreAMP Pathway Primer Mix prior to profiling. miRNA profiling was performed with the with the Serum & Plasma miScript miRNA PCR Array. Plots of **A** mean $C_T$ values achieved and **B** number of miRNAs detected demonstrate highly superior results from 10 fold less starting cDNA due to preamplification.

Some extracellular circulating miRNAs appear to be restricted to exosomes. Exosome enrichment traditionally requires an ultracentrifugation step to pellet the exosomes. This pellet can then be processed with the miRNeasy Serum/Plasma Kit. There are several alternative methods to enrich for exosomes, but the reproducibility of these methods is as yet undetermined. Nevertheless, the miRNeasy Micro Kit and miRNeasy Serum/Plasma Kit can be used for RNA isolation after polymer precipitation, immunocapture, or other methods to enrich or purify exosomes.

Urine is a body fluid that also shows promise for biomarker discovery, particularly for diseases of the kidney and prostate. The amount of miRNA recoverable from 200 µl urine is usually not enough for a 96-assay or 384-assay PCR array. Processing a much larger sample would help increase RNA yield, but would also cause increased copurification of any inhibitors present in the sample. For analysis of cellular miRNA, cells and debris should be pelleted and the pellet should then be used for purification with the miRNeasy Micro Kit. For analysis of cell-free miRNA from urine, we recommend removal of cells ▶

and cell debris by performing a pre-clearing spin or filtration. For cell-free miRNA purification, we recommend processing 100–200 µl urine using the miRNeasy Serum/Plasma Kit, followed by cDNA synthesis using 1.4 µl eluate, and preamplification using 1 µl cDNA synthesis reaction. Preamplification enables practical and robust miRNA profiling from human, mouse, or rat urine (Figure 9).

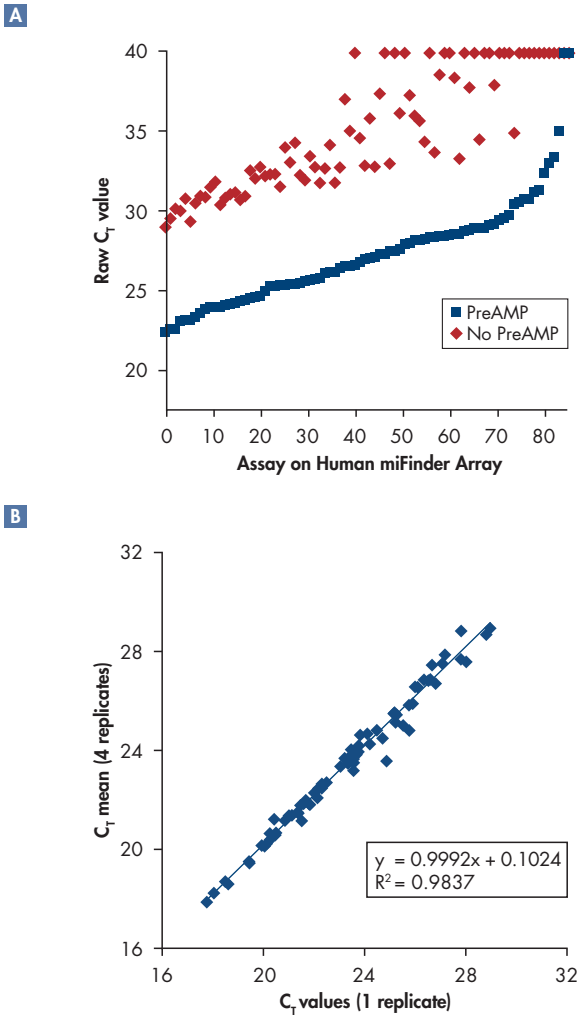


**Figure 9. Preamplification enables miRNA profiling from urine.** Total RNA was purified from 200 µl urine using the miRNeasy Serum/Plasma Kit. cDNA was then prepared from 1.5 µl using the miScript II RT Kit with miScript HiSpec Buffer. cDNA was either used directly for profiling or preamplified using the miScript PreAMP PCR Kit with Human miFinder miScript PreAMP Pathway Primer Mix. Profiling was performed with the Human miFinder miScript miRNA PCR Array. **A** $C_T$ values show that preamplification provides reliable expression data from miRNAs undetectable in nonpreamplified samples. **B** A scatter plot shows the high correlation in mean $C_T$ values achieved between 4 RNA isolations from urine and an individual RNA isolation from urine.

Our initial experiments with other body fluids, including cerebrospinal fluid, milk, and bronchial lavage suggest that the yield of RNA depends on whether the sample comes from a healthy or disease sample and on the extent of cellular content in the sample. For exosomal and extracellular RNA, a clarifying spin is always required to remove cells and cellular debris.

For small amounts of cultured cells, sorted cells, and laser capture microdissection (LCM) samples from cryosections, as well as various animal and human tissues, we recommend the miRNeasy Micro Kit, which is specifically designed for purification of total RNA, including miRNA, from small samples.

If after miRNA purification, the RNA content of the eluate is not known, QIAGEN offers a prespotted miRNA control plate, the miScript miRNA QC PCR Array, as a useful aid to determine whether preamplification is required or how much the preamplified sample needs to be diluted. Following the protocol provided with this array, it is straightforward to determine whether there is sufficient miRNA in the sample or whether a preamplification step is warranted (Figure 10). In addition, this control array can be used to determine whether inhibitors are present in the samples, making it a useful tool for quality control prior to performing a pathway or miRNome experiment.

## Normalization control for cell-free miRNA

Small, noncoding RNAs, such as snRNAs and snoRNAs, are frequently used for normalization of miRNA expression data. However, these RNAs are not expressed in serum and plasma and for this reason alternative methods of normalization are necessary in experiments involving these sample types. We recommend spiking a synthetic RNA into the sample prior to RNA purification. The spiked-in RNA can be later detected and this data used to normalize for differences in recovery during the purification procedure and differences in amplification efficiency. QIAGEN provides the miRNeasy Serum/Plasma Spike-In Control for this purpose. This synthetic RNA is amplified during the preamplification procedure and an assay to detect this RNA is provided in the miRNeasy Serum/Plasma Kit and on the miScript miRNA QC PCR Array.

Following calibration for differential RNA recovery, the global $C_T$ mean of commonly expressed targets (for miRNome and pathway expression profiling) or the $C_T$ mean of invariant miRNAs (for small panel expression profiling) can be used for qRT-PCR data normalization (21, 22).

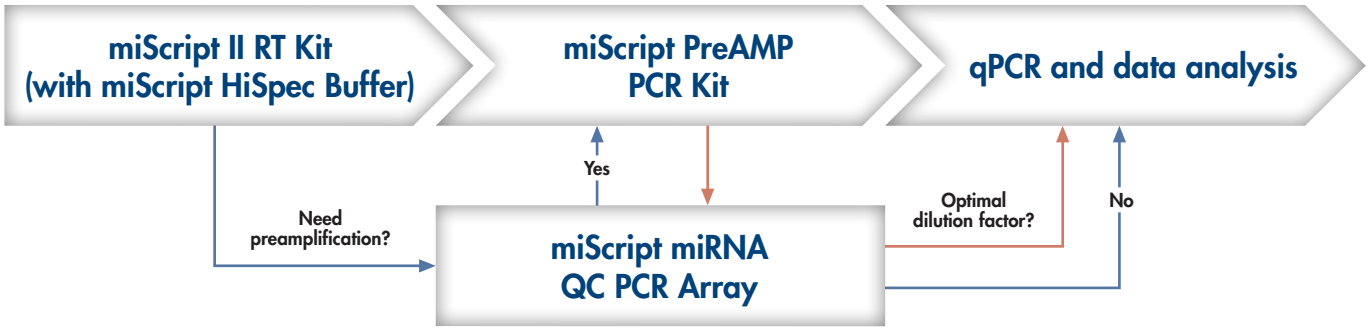


**Figure 10. Workflow for samples of unknown RNA amount and quality.** Control assays included in the miScript PreAMP PCR Kit and on the miScript miRNA QC PCR Array can be used to determine the need for preamplification and the optimal dilution factor for preamplified cDNA, and the presence or absence of PCR inhibitors.

## Conclusion

miRNA biomarker discovery research presents major challenges due to the fact that the very samples that are the most promising are also those that may be in shortest supply and have very low RNA content. At the same time, the discovery process requires screening for large numbers of miRNAs with a wide range of expression levels in multiple replicates. In a single step, preamplification can overcome these issues by providing reliable, nonbiased, highly multiplex amplification of miRNAs in a sample. Preamplification has been integrated into the miScript PCR System for miRNA quantification by qRT-PCR. Combined with specialized miRNeasy Kits for miRNA purification, this enables complete miRNA biomarker discovery experiments. Preamplification allows researchers to uncover previously inaccessible miRNA expression data. This will undoubtedly add to the already extraordinary discoveries identifying how these small molecules contribute to disease and cell biology, and facilitate the practical application of miRNA expression profiles to diagnostics and drug development.

### References


1. Lee, R.C., Feinbaum, R.L., and Ambros, V. (1993) The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell **75**, 843.
2. Shabalina, S.A. and Koonin, E.V. (2008) Origins and evolution of eukaryotic RNA interference. Trends in Ecology and Evolution **10**, 578.
3. Brodersen, P. et al. (2008) Widespread translational inhibition by plant miRNAs and siRNAs. Science **320**, 1185.
4. He, L. and Hannon, G.J. (2004) microRNAs: small RNAs with a big role in gene regulation. Nature **5**, 522.
5. Lee, Y. et al. (2004) microRNA genes are transcribed by RNA polymerase II. EMBO J. **23**, 4051.
6. Chen, K. and Rajewsky, N. (2007) The evolution of gene regulation by transcription factors and microRNAs. Nature Reviews Genetics **8**, 93.
7. Bartel, D.P. (2009) microRNAs: target recognition and regulatory functions. Cell **136**, 215.
8. Cai, X., Hagedorn, C.H., and Cullen, B.R. (2004) Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA **10**, 1957.
9. Zhou, X. et al. (2007) Characterization and identification of microRNA core promoters in four model species. PLoS Comput. Biol. **3**, e37.
10. Kim, Y.K. and Kim, V.N. (2007) Processing of intronic microRNAs. EMBO J. **26**, 775.
11. Iorio, M.V. et al. (2005) microRNA gene expression deregulation in human breast cancer. Cancer Res. **65**, 7065.
12. Mitchell, P.S. et al. (2008) Circulating microRNAs as stable blood-based markers for cancer detection. Proc. Natl. Acad. Sci. USA **105**, 10513.
13. Chim, S.S. et al. (2008) Detection and characterization of placental microRNAs in maternal plasma. Clin. Chem. **54**, 482.
14. Arroyo, J.D. et. al. (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma, Proc. Natl. Acad. Sci. USA **108**, 5003.
15. Vickers, K.C. et. al. (2011) MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat. Cell Biol. **13**, 423.
16. Wang, K. et al. (2010) Export of microRNAs and microRNA-protective protein by mammalian cells. Nucleic Acids Res. **38**, 7248.
17. Hunter, M.P. (2008) Detection of microRNA expression in human peripheral blood microvesicles. PLoS One **3**, e3694.
18. Ramachandran, S. and Palanisamy, V. (2012) Horizontal transfer of RNAs: exosomes as mediators of intercellular communication. WIREs RNA 2012 **3**, 286.
19. Kogure, T. et al. (2011) Intercellular nanovesicle-mediated microRNA transfer: a mechanism of environmental modulation of hepatocellular cancer cell growth. Hepatology **54**, 1237.
20. Blow, M.J. et al. (2006) RNA editing of human microRNAs. Genome Biol. **7**, R27.
21. Mestdagh, P. et al. (2009) A novel and universal method for microRNA RT-qPCR data normalization. Genome Biology **10**, R64.
22. D'haene, B. et al. (2012) miRNA expression profiling: from reference genes to global mean normalization. Methods Mol. Biol. **822**, 261.

## Ordering Information

| Product | Contents | Cat. no. |
|---|---|---|
| miScript II RT Kit (12) | Reagents for 12 x 20 µl cDNA synthesis reactions | 218160 |
| miScript II RT Kit (50) | Reagents for 50 x 20 µl cDNA synthesis reactions | 218161 |
| miScript PreAMP PCR Kit (12) | HotStarTaq® DNA Polymerase, buffer, primers, and controls for 12 preamplification reactions | 331451 |
| miScript PreAMP PCR Kit (60) | HotStarTaq DNA Polymerase, buffer, primers, and controls for 60 preamplification reactions | 331452 |
| miScript PreAMP Pathway Primer Mix | 60 µl primer mix for preamplification; for use with a Pathway-Focused miScript miRNA PCR Array | Varies |
| miScript PreAMP miRNome Primer Mix | 60 µl/tube primer mix for preamplification; for use with a miRNome miScript miRNA PCR Array | Varies |
| miScript SYBR Green PCR Kit (200) | Reagents for 200 x 50 µl PCRs | 218073 |
| miScript SYBR Green PCR Kit (1000) | Reagents for 1000 x 50 µl PCRs | 218075 |
| miScript PCR Starter Kit | Reagents for 10 x 20 µl cDNA synthesis reactions and 40 x 50 µl PCRs | 218193 |
| miScript Primer Assay (100) | miRNA-specific primer for 100 x 50 µl PCRs | Varies* |
| Pathway-Focused miScript miRNA PCR Array | Pathway or disease panels of miRNA assays | 331221 |
| miRNome miScript miRNA PCR Array | miRNome panels of miRNA assays | 331222 |
| Custom miScript miRNA PCR Array | Custom panels of miRNA assays | 331231 |
| miRNeasy Micro Kit (50) | Columns, plasticware, and reagents for 50 preps | 217084 |
| miRNeasy Serum/Plasma Kit (50) | Columns, plasticware, and reagents for 50 preps | 217184 |
| miRNeasy Serum/Plasma Spike-In Control | 10 pmol *C. elegans* miR-39 miRNA mimic spike-in control for serum/plasma samples | 219610 |
| miRNeasy FFPE Kit (50) | Columns, plasticware, and reagents for 50 preps | 217504 |

* Visit GeneGlobe to search for and order these products (www.qiagen.com/GeneGlobe).

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

**For more information on QIAGEN's miRNA portfolio for biomarker discovery, visit www.qiagen.com/Serum-Plasma.**

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