

# A complete workflow for high-throughput isolation of serum miRNAs and downstream analysis by qRT-PCR: application for cancer research and biomarker discovery

## Abstract

Biomarkers have become invaluable clinical research tools for cancer detection. There is an urgent need for biomarkers that are more sensitive and specific predictors to help decrease complications and morbidity related to prostate biopsies, and circulating microRNA (miRNA) in blood could potentially be a solution. With this future goal in mind, we developed a complete workflow providing: (1) high-throughput isolation of total serum RNA using the Applied Biosystems™ MagMax™ *mirVana*™ Total RNA Isolation Kit for the Thermo Scientific™ KingFisher™ Flex or Duo instrument, with (2) downstream analysis of miRNA profiles by qRT-PCR using the Applied Biosystems™ TaqMan™ Advanced miRNA cDNA Synthesis Kit and TaqMan™ Advanced miRNA Assays. The effectiveness of this workflow is exemplified here by analysis of a panel of miRNAs derived from prostate cancer serum samples versus a normal sample control group. By following this fast and easy workflow that is suitable for serum and other body fluids, disease-specific RNA signatures can be identified and used as biomarkers.

## Introduction

An ideal biomarker should be easily assayed with minimally invasive procedures but possess high sensitivity and specificity. Despite many candidate biomarkers researched and proposed in the literature, very few have been able to meet these needs and translate over to clinical use. In fact, there are no reliable biomarker options available for certain types of cancer and other diseases. This highlights the growing need for more in-depth biomarker research and methods more amenable to greater sample throughput.

The use of one biomarker, prostate-specific antigen (PSA), has led to a dramatic increase in the diagnosis of prostate cancer—over 230,000 new cases in 2014 [1]. While it is a sensitive test, less than one-fourth of patients undergoing a prostate biopsy based on PSA testing are actually found to have the disease. The identification of more sensitive and specific biomarkers could be highly beneficial in clinical research and for potential future clinical utility.

Circulating RNA, and in particular serum miRNA, could potentially fill this void in the future, providing biomarkers more sensitive to detection. The main advantages of miRNAs versus longer molecules (mRNA, lncRNA) include: (1) a smaller number of sequences (<2,500) and thus ease of analysis and (2) stability due to small size (~16–27 nt) and thus robustness of detection. A number of serum miRNAs have been reported in the literature as potentially promising biomarkers of prostate cancer [2-4]. Here we describe a robust high-throughput workflow that enables highly sensitive and reproducible processing of clinical research sample types, including serum, to screen for miRNA biomarkers.

## Materials and methods

### RNA isolation

Total RNA (including miRNA) was isolated from 100 µL of serum from individuals with prostate cancer and from healthy individuals (in duplicate) using the MagMAX *mirVana* Total RNA Isolation Kit and the Thermo Scientific™ KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head.

## Analysis of miRNA targets

cDNA was generated using 2  $\mu$ L of purified total RNA with the TaqMan Advanced miRNA cDNA Synthesis kit. qPCR was then performed (in triplicate) for each sample using 2  $\mu$ L of diluted cDNA, TaqMan Advanced miRNA Assays, and Applied Biosystems™ TaqMan™ Fast Advanced Master Mix under fast cycling conditions.

## Analysis of mRNA targets

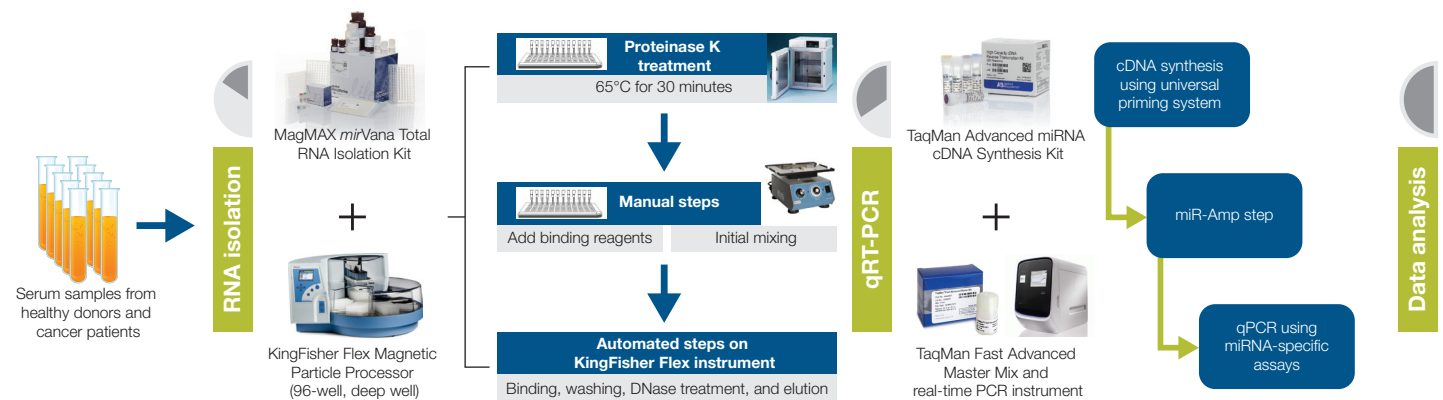
cDNA was generated using 10  $\mu$ L of total RNA with the Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit. qPCR was then performed (in triplicate) for each sample using 2  $\mu$ L of cDNA, TaqMan™ Assay specific for the reference mRNA target, and TaqMan Fast Advanced Master Mix under fast cycling conditions.

## Results

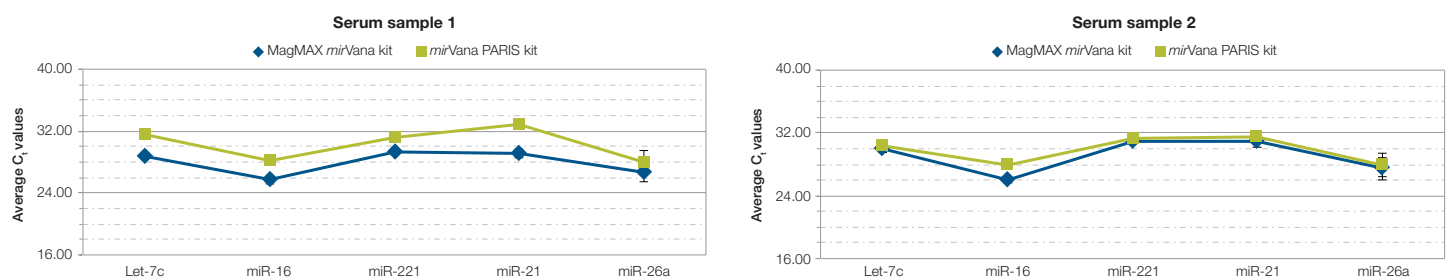
We have successfully developed a robust high-throughput workflow that enables processing of clinical research sample types, in particular serum,

to identify miRNA biomarkers using qRT-PCR methodology. The high-throughput isolation method (Figure 1, left) allows faster processing of samples by using MagMAX™ bead-based chemistry, with alternative upfront purification steps to replace organic extraction and the ability to run up to 96 samples at a time with the KingFisher Flex or Duo instrument. For downstream analysis of recovered RNA by qRT-PCR, the TaqMan Advanced miRNA cDNA Synthesis Kit uses a universal RT primer-based system to simplify the workflow and provide flexibility for content coverage (Figure 1, right). The included preamplification (miR-Amp) step allows universal amplification for greater sensitivity to low-copy targets.

The magnetic bead-based MagMAX *mirVana* Total RNA Isolation Kit offers superior performance and high-throughput capabilities for the RNA purification step. Figure 2 highlights performance of the MagMAX *mirVana* kit versus the Ambion™ *mirVana*™ PARIS™ Kit, which employs a filter-based organic extraction method widely used for isolating total RNA (including



**Figure 1. Workflow for high-throughput isolation of RNA from serum, and qRT-PCR analysis of miRNA.** The high-throughput RNA isolation method allows faster processing of samples by using bead-based chemistry and removing organic extraction steps. The TaqMan Advanced miRNA cDNA Synthesis Kit and TaqMan Advanced miRNA Assays simplify the workflow and provide flexibility for content coverage. An included miR-Amp step allows universal amplification for greater sensitivity to low-copy targets.



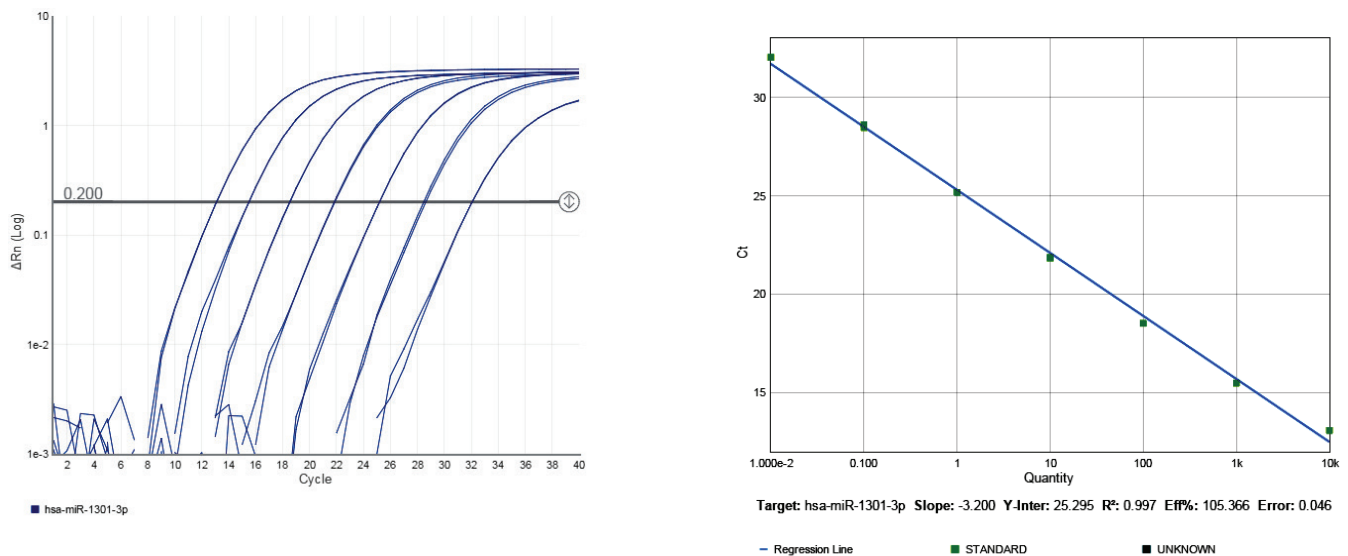
**Figure 2. The MagMAX *mirVana* kit offers superior performance and high-throughput capabilities compared to the widely used *mirVana* PARIS kit.** RNA was isolated from the serum of 2 normal samples, and levels of 5 miRNAs were measured by qRT-PCR. Greater sensitivity for each target was observed using RNA isolated with the MagMAX *mirVana* kit.

miRNA) from serum. In this experiment, RNA was isolated from the serum of two healthy individuals, then expression of 5 miRNAs was measured by qRT-PCR using the TaqMan Advanced miRNA Assays. The MagMAX *mirVana* kit demonstrates superior performance and high-throughput capabilities that are crucial for clinical research.

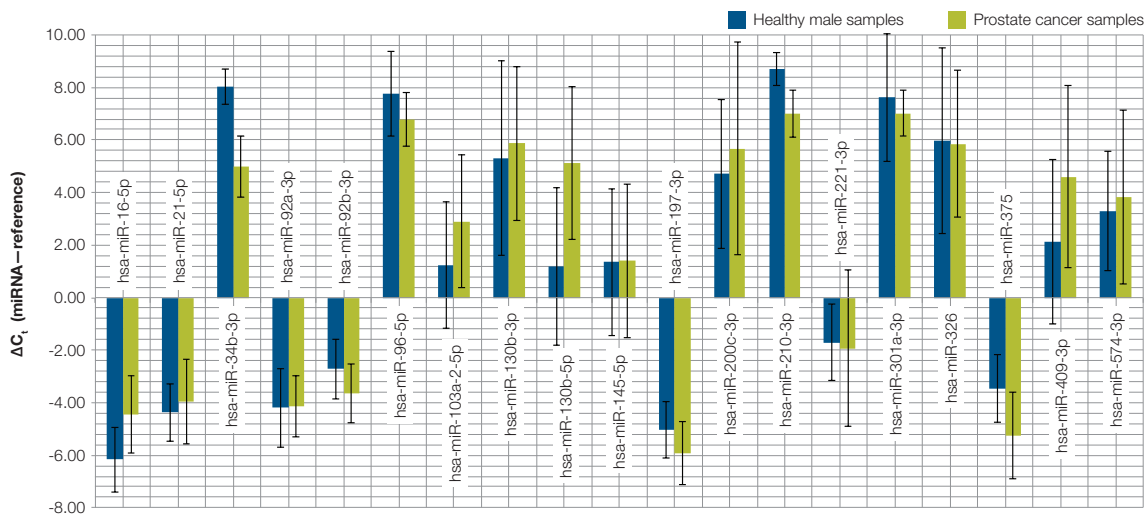
The TaqMan Advanced miRNA cDNA Synthesis Kit and TaqMan Advanced miRNA Assays feature excellent performance and sensitivity for the downstream qRT-PCR analysis of miRNA isolated from serum and other biofluids. Figure 3 shows an amplification plot and standard curve demonstrating 6-log linear dynamic range with input ranging from

60 to 60 million copies using a synthetic template. No background signals in reactions without template were observed.

We utilized the workflow described above for identification of blood-borne miRNA biomarkers for prostate cancer. Total RNA was isolated from 10 prostate cancer serum samples (PSA levels 5–100 ng/mL) and 10 normal samples. A panel of 19 miRNAs was identified from the literature [2-4] and previous in-house studies as potential serum biomarkers for prostate cancer. Levels of these 19 miRNAs were measured in serum-derived RNA for both study groups (Figure 4). Three miRNAs were present at significantly higher levels in the serum of



**Figure 3. Dynamic range and sensitivity.** Amplification plot and standard curve shows 6-log linear dynamic range with target ranging from 60 to 60 million copies.



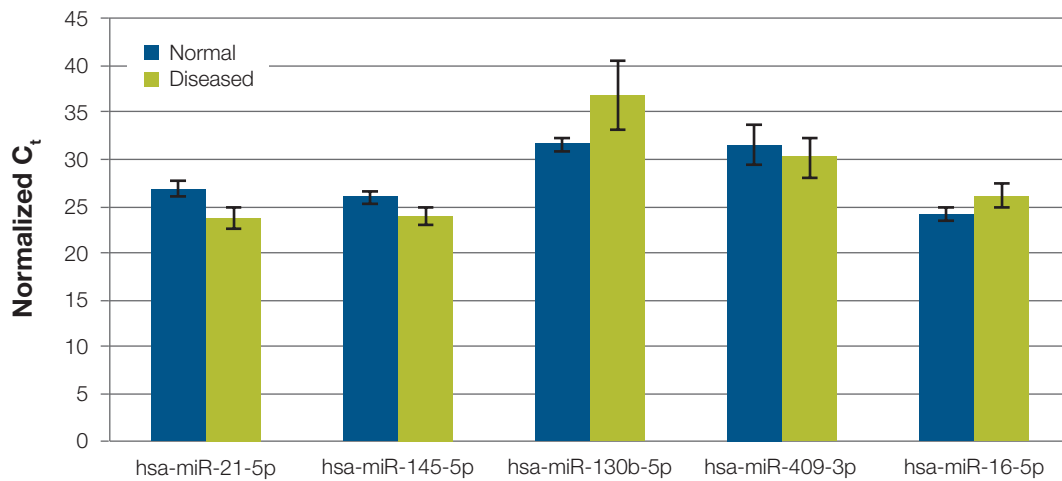
**Figure 4. Identification of miRNA biomarker candidates for prostate cancer.** Total RNA was isolated from the serum of 10 individuals with prostate cancer and 10 healthy individuals using the MagMax *mirVana* Total RNA Isolation Kit. Expression levels of 19 miRNAs were measured by qRT-PCR using the TaqMan Advanced miRNA cDNA Synthesis Kit and TaqMan Advanced miRNA Assays. The y-axis displays  $\Delta C_t$  values for each miRNA, normalized to reference mRNA.

the individuals with prostate cancer: hsa-miR-210-3p ( $P < 0.0001$ ), hsa-miR-34b-3p ( $P < 0.0001$ ), and hsa-miR-375 ( $P = 0.0277$ ). These miRNAs represent a set of differentially expressed biomarkers that could have potential clinical research utility for prostate cancer.

The study presented here uses an mRNA reference for normalization of the  $\Delta C_t$  values for each miRNA. However, normalization methods using exogenous spike-in controls are becoming more widely accepted for normalizing miRNA qRT-PCR data from serum and other body fluids. In a separate study, we also demonstrated the use of cel-miR-54 as a spike-in control for serum samples (Figure 5).

## Conclusions

We have developed a workflow with a set of reagents and tools for recovery and analysis of circulating miRNAs from blood samples. The complete workflow includes high-throughput isolation of total serum RNA and sensitive, reproducible downstream analysis of miRNA by qRT-PCR. Using this workflow, 19 miRNAs were screened to identify potential serum biomarkers for prostate cancer. Three miRNAs were expressed at significantly higher levels in the prostate cancer samples versus the normal sample group. This study demonstrates that the workflow can be used for the investigation of novel miRNA biomarkers in serum samples. With appropriate TaqMan Assays, the workflow may also be extended for the analysis of other potential RNA biomarkers in cancer research.



**Figure 5. Synthetic spike-in exogenous controls as reliable normalization tools for miRNAs from serum.**  $C_t$  values were median normalized against cel-miR-54 [5].

## Acknowledgments

We would like to thank Alex J. Rai, from the Department of Pathology and Cell Biology and Center for Advanced Laboratory Medicine at Columbia University Medical Center and New York Presbyterian Hospital, and Joel DeCastro, from the Department of Urology at Columbia University Medical Center and New York Presbyterian Hospital, for their contributions to this research.

## Ordering information

Product	Cat. No.
TaqMan Advanced miRNA Assays	A25576
TaqMan Advanced miRNA cDNA Synthesis Kit	A28007
MagMax <i>mirVana</i> Total RNA Isolation Kit	A27828
TaqMan Fast Advanced Master Mix	4444557
KingFisher Flex Magnetic Particle Processor with 96 Deep-Well Head	5400630

## References

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All donor samples used in this study were procured for research use only.

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