miRNA from serum and plasma samples

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Introduction

microRNAs (miRNAs) have shown potential in biomarker discovery and diagnostic screening. Circulating miRNAs are present in serum and plasma, which are easier and less invasive to collect than traditional tissue biopsies.

Because of the presence of circulating miRNAs, there is increasing interest in serum and plasma as "liquid biopsy" samples in disease and cancer research. They can provide a sensitive method to discover and monitor diseases.

miRNA:

- Contains few nucleotides.
- Is relatively stable due to interactions with protective mechanisms, including exosomes, extracellular vesicles, and protein complexes.

Due to the potential for variability, several factors must be considered when studying miRNA from serum or plasma. These factors include:

- Sample type (serum or plasma)
- Sample collection and handling
- Sample storage
- RNA and miRNA extraction
- RNA and miRNA quantity and quality
- Methods to analyze expression

Consideration of these factors helps to ensure that the RNA expression pattern remains stable and originates from the target sample and not other components of whole blood. This Reference Guide discusses these potential variables and provides suggestions.

Use of serum or plasma

There is debate over whether serum or plasma is the most appropriate sample type to detect both miRNA and RNA expression.

Several studies have found that plasma samples provide a higher recovery of miRNA (based on real-time RT-PCR), although others have seen no or minimal difference between serum and plasma (Kroh EM et al., 2010; McDonald JS et al., 2011; Wang K et al., 2012).

We have found that both serum and plasma samples work well for miRNA and RNA detection but that recovery is slightly higher from plasma samples (Figure 1).

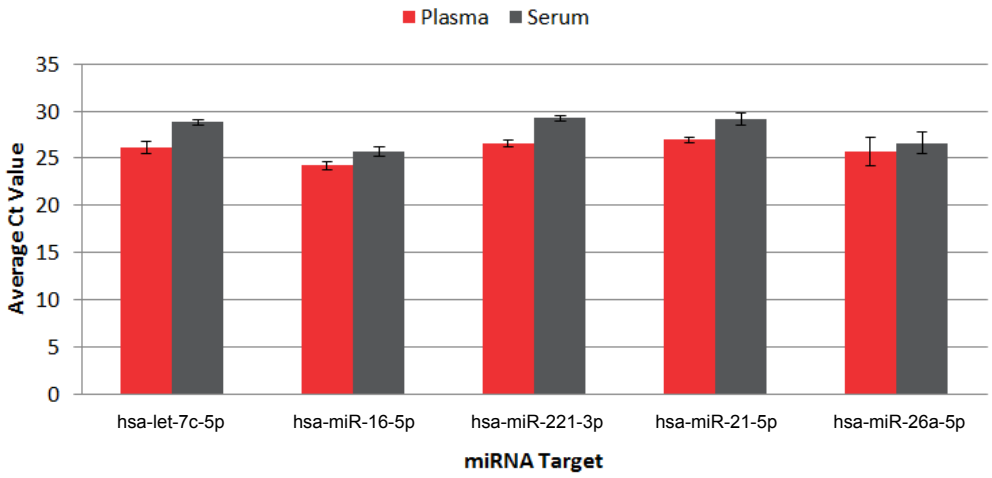

Figure 1 Comparison of miRNA expression in plasma and serum

RNA was extracted from 100 µL of human plasma (K2–EDTA) or 100 µL of human serum using the MagMAX™ *mir*Vana™ Total RNA Isolation Kit (Cat. No. A27828). miRNA was analyzed by real-time PCR using the TaqMan® Advanced miRNA cDNA Synthesis Kit (Cat. No. A28007) and TaqMan® Advanced miRNA Assays (n=6, ±1 standard deviation).

Although the consistency of the sample type (serum or plasma) between studies is important to consider, ultimately, proper sample collection and storage are the critical factors for miRNA recovery and analysis.

Sample collection and handling

There are several variables in sample collection and handling that can affect RNA recovery and its use in downstream analysis:

- The collection tube, including the type of additive
- The sample volume
- Sample handling, including any pre- and post-processing steps
- Sample centrifugation

Collection tubes

Standard collection for both serum and plasma is done using BD™ Vacutainer® Blood Collection Tubes. These tubes are available with a range of different additives, depending on the needs of the user.

See **education.bd.com/images/view.aspx?productId=1532**.

Table 1 Collection tubes

Sample type	Tube properties	Usage notes
Whole blood for serum samples	• Do not contain anticoagulants. • Use co-activators to separate the serum from the other components of the whole blood.	—
Whole blood for plasma samples	Tubes treated with: • EDTA • Citrate • Citrate derivatives	• Most commonly available. • Our recommendation for RNA applications.
	Tubes treated with heparin.	Used less frequently for nucleic acid applications due to the potential for inhibition of PCR.
	Tubes with other coating (for example, sodium polyanethol sulfonate).	Less commonly available and are not used for nucleic acid applications.
Whole blood collection for both serum and plasma samples.	Tubes with a fixative or preservative.	• Preserve the expression pattern during storage. • Can be expensive. • Still require strict handling guidelines to prevent variation during collection. • More difficult to handle for nucleic acid extraction.

Sample collection volume

The volume of the sample that is collected is important to maximize the amount of material available for RNA extraction and for proper clotting or anti-coagulation. Insufficient amounts result in poor storage stability and even in the carry-over of inhibitors in downstream analysis.

Collect the volume of sample recommended by the sample collection tube.

Sample handling and processing

Proper sample handling ensures that differences that are detected between sample types are due to true differences and not a result of variability in handling. A standard process ensures that all factors are constant and consistent. An example of a standard process is *Early Detection Research Network (ERDN) Standard Operating Procedure (SOP) for collection of EDTA plasma and serum* (Tuck MK et al., 2009).

Pre-processing steps, including incubations, mixing the contents of the collection tube, and phase separation by centrifugation, are critical to serum and plasma stability. These steps help prevent hemolysis (the lysis of red blood cells) (Tyndall L and Innamorato S, 2004). Pre-processing methods differ depending on sample type. Therefore, it is essential to follow or develop a standard process for sample collection and storage.

Sample centrifugation

Serum and plasma can be further centrifuged to remove any remaining cells after the post-processing collection steps are complete. This centrifugation step is not required and depends on the target gene expression. For example, cellular debris must be centrifuged and removed if targeting RNA expression in exosomes or extracellular vesicles. Otherwise, the cellular debris can bias the analysis for cellular RNA expression.

Complete all additional centrifugation steps before freezing the sample. Freezing the sample before centrifugation lyses the unwanted cells and prevents their separation out of the sample.

Sample storage

Divide samples into smaller aliquots before freezing to prevent freeze-thaw cycles of each sample. Multiple freeze-thaw cycles can cause RNA degradation and potentially change the expression pattern of the miRNA and RNA. We recommend no more than one freeze-thaw cycle as a precaution.

The following storage temperatures and times help to prevent RNA degradation and preserve the expression pattern of the miRNA and RNA:

Temperature	Time
4°C	Less than 8 hours, not recommended for long-term storage
–20°C	Less than one week
–80°C	More than one week

Extraction of RNA

Flexible and sensitive extraction techniques are required due to:

- The cell-free nature of serum and plasma.
- The low abundance of RNA in serum and plasma samples.
- The smaller size of miRNA relative to other types of RNA.
- The different forms of miRNA (contained in exosomes or as part of protein complexes).

Most RNA in serum and plasma is contained in protein complexes or extracellular vesicles (for example, exosomes). Strong lysis is required to release the RNA for extraction.

In addition to association with proteins and extracellular vesicles, serum and plasma samples also contain free-floating RNA (cell-free RNA or cfRNA). There is only a small amount of this cfRNA and it is not of concern for lysis.

Table 2 Options for strong lysis

Extraction method	Properties	Reagent or method
Enzymatic treatment	• Releases and protects the RNA. • Can be combined with magnetic bead-based purification, which is compatible with: – High-throughput sample processing. – Automated sample processing.	Proteinase K digestion
Organic compound extraction	• Releases and protects the RNA. • Works best with: – Filter-based purification columns. – Ethanol precipitation. • Limits the user to a lower number of samples than enzymatic treatment. • Requires more handling than enzymatic treatment.	TRIzol™ Reagent Phenol-chloroform extraction

Table 3 Available kits

Extraction method	Kit	Cat. No.
Enzymatic treatment	MagMAX™ *mir*Vana™ Total RNA Isolation Kit	A27828
Organic compound extraction	*mir*Vana™ PARIS™ RNA and Native Protein Purification Kit	AM1556

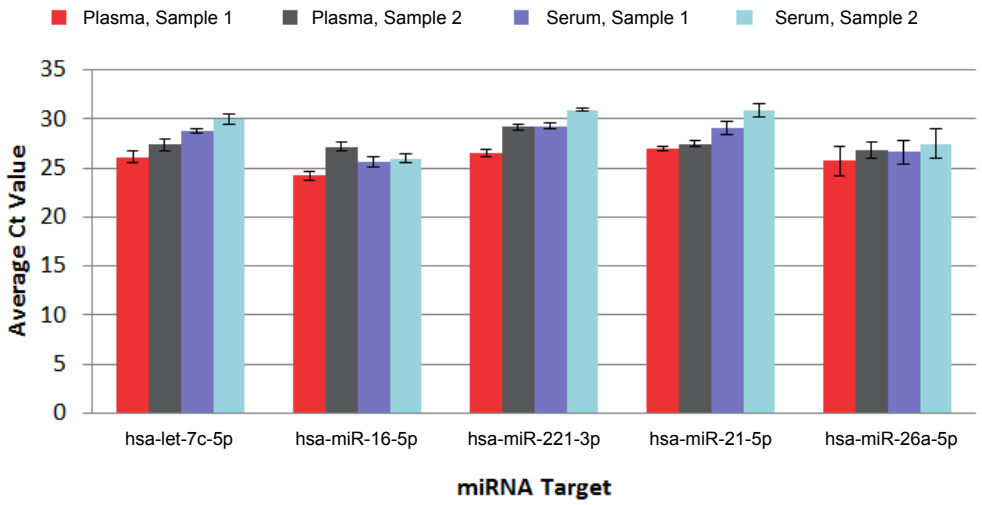

Figure 2 miRNA expression after RNA extraction from serum and plasma with MagMAX™ *mir*Vana™ Total RNA Isolation Kit

RNA was extracted from 100 µL of human plasma (K2–EDTA) or 100 µL of human serum from two samples using the MagMAX™ *mir*Vana™ Total RNA Isolation Kit (Cat. No. A27828). miRNA was analyzed by real-time RT–PCR using the TaqMan® Advanced miRNA cDNA Synthesis Kit (Cat. No. A28007) and TaqMan® Advanced miRNA Assays (n=6, ±1 standard deviation).

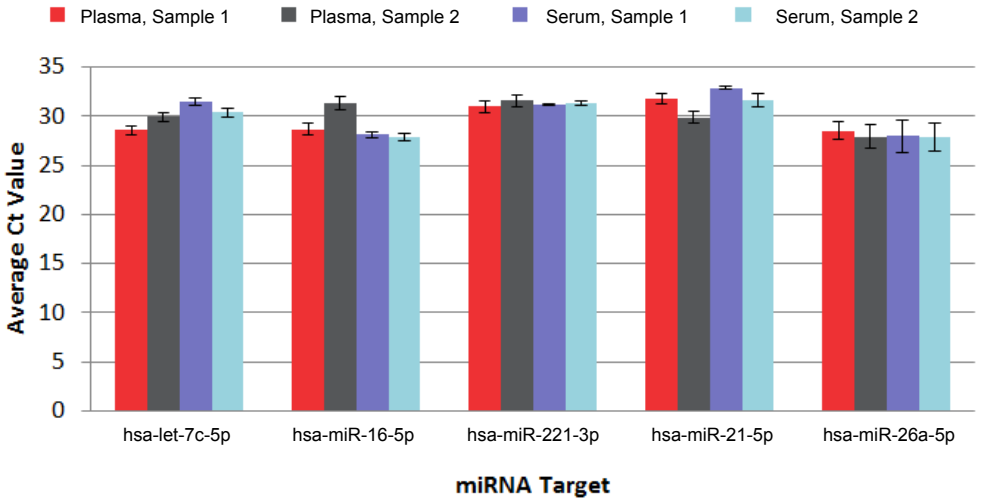

Figure 3 miRNA expression after RNA extraction from serum and plasma with *mir*Vana™ PARIS™ RNA and Native Protein Purification Kit

RNA was extracted from 100 µL of human plasma (K2–EDTA) or 100 µL of human serum from two samples using the *mir*Vana™ PARIS™ RNA and Native Protein Purification Kit (Cat. No. AM1556), with the plasma/serum protocol. miRNA was analyzed by real-time RT–PCR using the TaqMan® Advanced miRNA cDNA Synthesis Kit (Cat. No. A28007) and TaqMan® Advanced miRNA Assays (n=6, ±1 standard deviation).

Workflow for enzymatic treatment

Combine serum or plasma with an RNA-friendly digestion buffer and Proteinase K

▼

Incubate for 30 minutes at an elevated temperature (55–60°C)

▼

Add binding buffer and alcohol

▼

Bind to a solid phase (Silica column or beads)

▼

Wash with wash buffers that contain alcohol

▼

Elute into an optimized elution solution

Workflow for organic extraction

Combine serum or plasma with lysis buffer and organic solution

(TRIzol™ Reagent
Or
Guanidinium thiocyanate and acidic phenol–chloroform solution)

▼

Vortex, then centrifuge

▼

Collect the clear aqueous top layer

▼

Combine the aqueous layer with alcohol

▼

Bind to a solid phase (silica column or beads)
Or
Perform ethanol precipitation

▼

Wash with wash buffers that contain alcohol

▼

Elute into an optimized elution solution

RNA quality and quantity

We have found that real-time PCR is the simplest and most reliable way to examine RNA presence and quantity from serum and plasma samples. A first screen with housekeeping genes provides a snapshot of the amount of RNA and provides confidence in the quality to proceed with more detailed analysis, including OpenArray™ panels or RNA-Seq sequencing. Housekeeping genes include miR–16 and let–7e.

Traditional RNA quality testing methods are not as effective for RNA or miRNA from serum and plasma because of the low abundance of RNA. Traditional RNA quality testing methods include spectrophotometry or fluorescence spectroscopy with NanoDrop™ instruments, fluorometric quantification with Qubit™ instruments, and electrophoresis with Agilent™ Bioanalyzer instruments.

Agilent™ Bioanalyzer instruments can provide an indication of RNA or miRNA quality from serum and plasma. If the overall quality of the RNA is good, it indicates that the miRNA is intact. A peak near the beginning of the run, the small RNA peak, indicates good-quality miRNA.

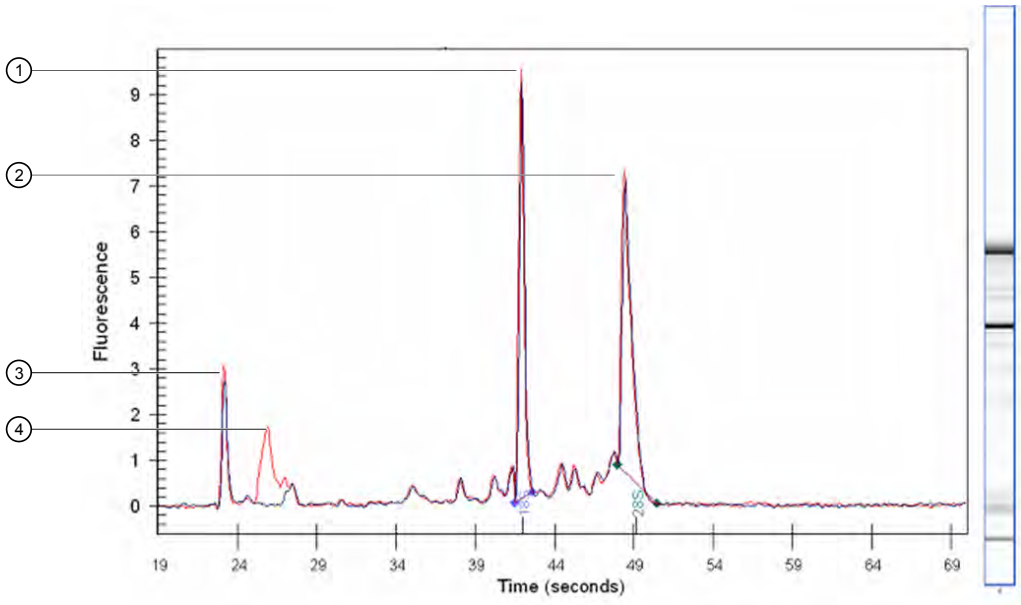

Figure 4 Scan of high-quality total RNA with miRNA from cultured cells with the Agilent™ Bioanalyzer

Total RNA was isolated from cultured cells using the MagMAX™ *mir*Vana™ Total RNA Isolation Kit.

1. 18S peak (not always visible in a scan of RNA from serum or plasma because of the relatively small amount of RNA)
2. 28S peak (not always visible in a scan of RNA from serum or plasma because of the relatively small amount of RNA)
3. Agilent™ Bioanalyzer size marker
4. Small RNA peak

Table 4 miRNA reported to have stable expression in serum and plasma

miRNA name	TaqMan® Advanced miRNA Assay
hsa-miR-24	477992_mir
hsa-miR-484	478308_mir
hsa-miR-93-5p	478210_mir
hsa-miR-191-5p	477952_mir
hsa-miR-126-3p	477877_mir
hsa-miR-16-5p	477860_mir

Case studies

miRNA biomarker research is a very powerful tool in understanding disease, for example cancer. This research can be enhanced by the ability to screen a large number of samples.

The following figures highlight the difference in miRNA expression in plasma from research samples without cancer and research samples with different cancers.

Total RNA was extracted from 100 µL of plasma from 12 human samples using the MagMAX™ *mir*Vana™ Total RNA Isolation Kit. Five research samples did not have cancer and 7 research samples had various cancers, including non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), and prostate cancer. Total RNA was converted to cDNA using the TaqMan® Advanced miRNA cDNA Synthesis Kit. Total RNA was analyzed using TaqMan® Advanced miRNA Assays on TaqMan® OpenArray™ Plates, which contain >700 miRNA targets.

The following data demonstrate the power of high-throughput extraction and analysis. The data show:

- The overall expression trends between normal and cancer research samples (Figure 5 on page 11).
- Focused results for miRNA groups (Figure 6 on page 12, Figure 7 on page 12, Figure 8 on page 13).

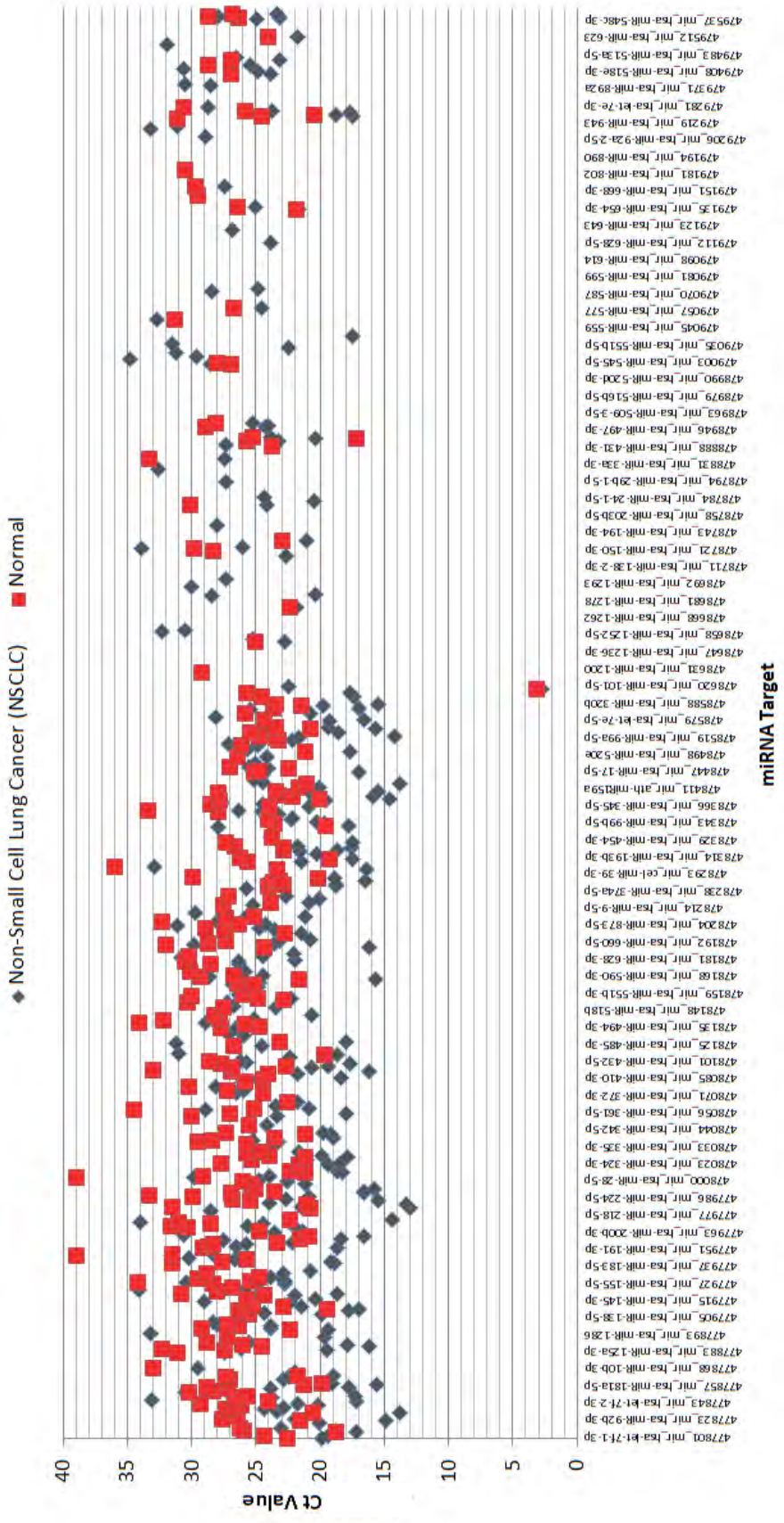

Figure 5 Complete data set for miRNA expression in normal and NSCLC research samples

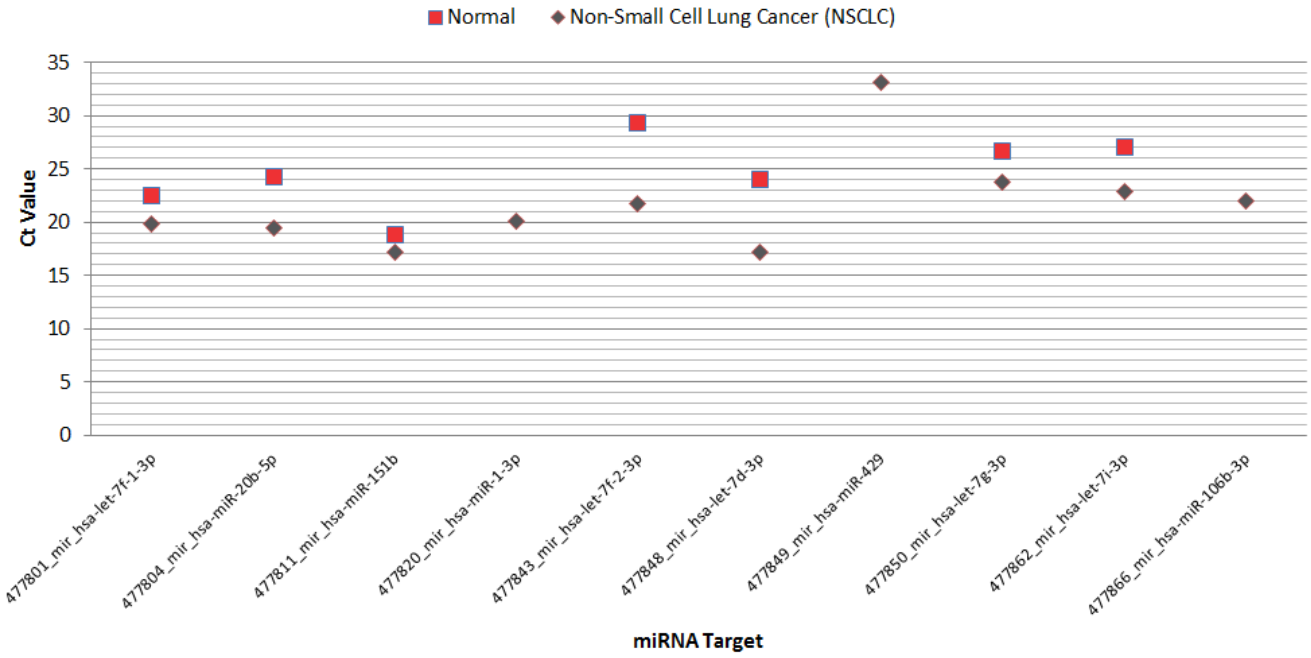

Figure 6 Select targets for miRNA expression in normal and NSCLC research samples

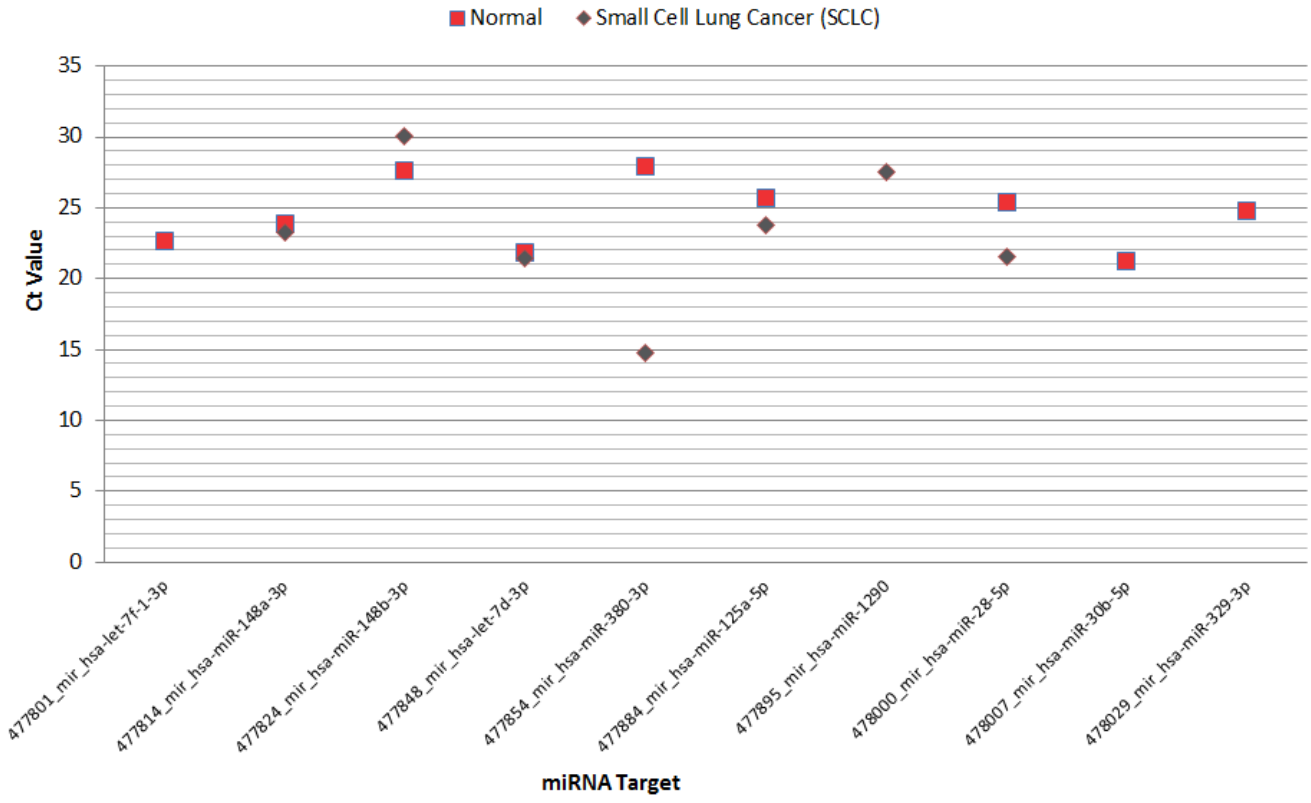

Figure 7 Select targets for miRNA expression in normal and SCLC research samples

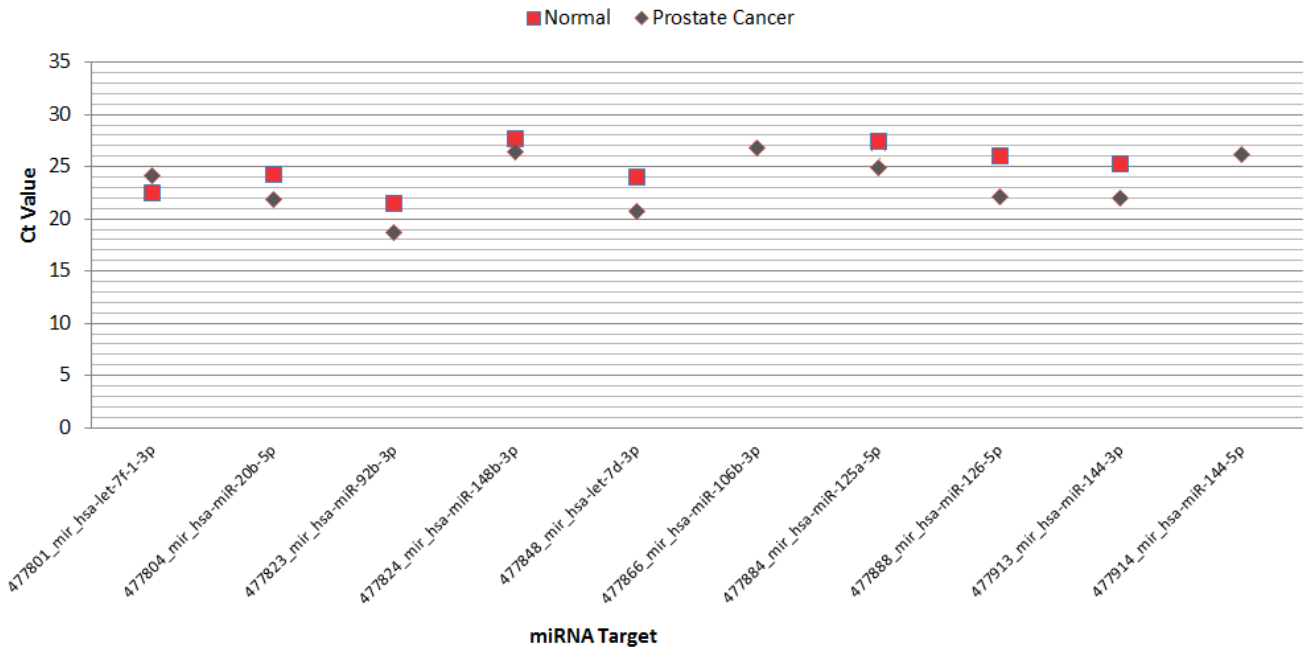

Figure 8 Select targets for miRNA expression in normal and prostate cancer research samples

Related documentation

Document	Pub. No.	Description
Application notes		
Simultaneous detection of miRNA and mRNA on TaqMan® Array Cards using the TaqMan® Advanced miRNA workflow	COL32016 1117	Describes expression patterns of miRNAs and mRNAs from serum samples on TaqMan® Array Cards and provides a protocol to detect miRNAs and mRNAs from a single reverse transcription reaction using the TaqMan® Advanced miRNA cDNA Synthesis Kit.
A complete workflow for high-throughput isolation of serum miRNAs and downstream analysis by qRT-PCR: application for cancer research and biomarker discovery	CO210328 0615	Describes a complete workflow to isolate and analyze circulating miRNA, including potential serum biomarkers for prostate cancer.
A technical guide to identifying miRNA normalizers using TaqMan® Advanced miRNA Assays	COL31302 0916	A compiled list of recommended endogenous in various tissues and biofluids. Includes an overview of protocols to verify miRNAs as real-time PCR normalizers.
User guides		
MagMAX™ mirVana™ Total RNA Isolation Kit (manual extraction) User Guide	MAN0011131	—
MagMAX™ mirVana™ Total RNA Isolation Kit (serum and plasma samples) User Guide (For high-throughput isolation)	MAN0011134	—
mirVana™ PARIS™ Kit Protocol	1556M	—

References

Kroh EM, Parkin RK, Mitchell PS, Tewari M (2010) Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription–PCR (qRT–PCR). *Methods* 50(4):298–301.

McDonald JS, Milosevic D, Reddi HV, et al. (2011) Analysis of circulating microRNA: Preanalytical and analytical challenges. *Clinical Chemistry* 57(6):833–840.

Tuck MK, Chan DW, Chia D, et al. (2009) Standard operating procedures for serum and plasma collection: early detection research network consensus statement *Standard Operating Procedure Integration Working Group*. *Journal of Proteome Research* 8(1):113–117.

Tyndall L, Innamorato S (2004) Managing preanalytical variability in hematology. *Lab Notes* 14(1):1–7.

Wang K, Yuan Y, Cho J-H, McClarty S, et al. (2012) Comparing the microRNA spectrum between serum and plasma. *PLOS One* 7(7):e41561.

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The information in this guide is subject to change without notice.

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Revision	Date	Description
A.0	2 March 2018	New document.

thermofisher.com/support | **thermofisher.com/askaquestion**

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