*mir*Vana™ qRT-PCR miRNA Detection Kit

Part Number AM1558

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Protocol

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I. Introduction

A. Product Description

The *mir*Vana™ qRT-PCR miRNA Detection Kit is a quantitative reverse transcription-PCR (qRT-PCR) kit enabling sensitive, rapid quantitation of microRNA (miRNA) expression from total RNA samples (Figure 1 on page 2). The assay is rapid and sensitive, and, unlike commonly used methods for detection of miRNAs, it is nonisotopic. miRNAs are small, single-stranded, ~19–23 nt RNA molecules encoded in the genomes of plants, animals, and viruses. Mature miRNAs enter the RNA-induced silencing complex (RISC) and guide the RISC to induce translational repression or endonucleolytic cleavage of specific target mRNAs.

Designed for use with *mir*Vana qRT-PCR Primer Sets, the *mir*Vana qRT-PCR miRNA Detection Kit reagents are optimized to amplify the specific miRNA targeted by the *mir*Vana qRT-PCR Primer Set used in the reaction. The kit includes the hsa-miR-24 qRT-PCR Primer Set for amplification of the widely expressed miR-24 from either the Human Heart Total RNA supplied with the kit or from user-supplied human, mouse, and rat RNA samples (Figure 2).

*mir*Vana qRT-PCR Primer Sets available for other targets each include a primer for reverse transcription and a PCR primer pair optimized for sensitive detection of specific miRNAs by qRT-PCR. For a complete listing of available Primer Sets, see: www.ambion.com/miRNA/primers.

Primer Sets that amplify conserved regions of U6 snRNA (P/N AM30303) and 5S rRNA (P/N AM30302) are available to normalize for RNA content among different experimental samples.

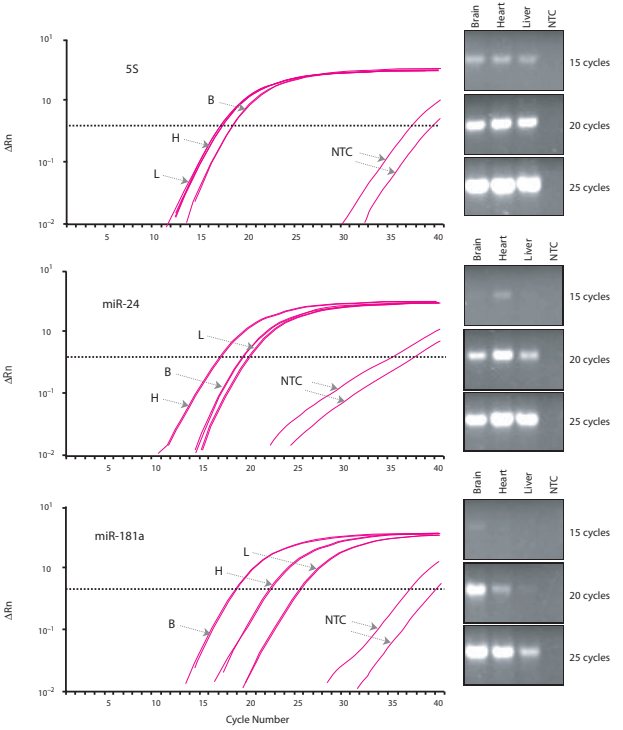

Figure 1. *mir*Vana™ qRT-PCR miRNA Detection Kit Results.

FirstChoice® Total RNA (25 ng) from human brain (B), heart (H), and liver (L) was tested for expression of the indicated small RNA species using the *mir*Vana qRT-PCR miRNA Detection Kit and the corresponding Primer Sets. Experiments were conducted using both real-time and end-point PCR. NTC: No template control.

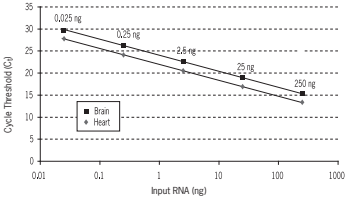

Figure 2. Linear Amplification of miR-24.

The indicated amounts of FirstChoice® Total RNA from human heart and brain were used to detect miR-24 using the *mir*Vana qRT-PCR miRNA Detection Kit. The C_t values shown are the average of duplicate reactions. These data illustrate the sensitivity and linearity of the *mir*Vana qRT-PCR miRNA Detection system; miR-24 was detected using only 25 pg of input RNA, and the dynamic range of the experiment was at least 5 logs.

B. Reagents Provided and Storage Conditions

When purchased with a *mir*Vana qRT-PCR Primer Set, the kit includes reagents for 200 RT-PCRs; including up to 100 reactions targeting the hsa-miR-24 positive control target. Note that thermostable DNA polymerase is not supplied with the kit. ***Do not store in a frost-free freezer.***

Amount	Component	Storage
440 µL	*mir*Vana™ 5X RT Buffer	–20°C
1.1 mL	*mir*Vana 5X PCR Buffer	–20°C
88 µL	ArrayScript™ Enzyme Mix*	–20°C
22 µL	Human Heart Total RNA (25 ng/µL)	–20°C
10 µL	10X hsa-miR-24 RT Primer	–20°C
11 µL	hsa-miR-24 PCR Primers	–20°C
2 x 1.75 mL	Nuclease-free Water	any temp†

* Contains ArrayScript™ reverse transcriptase and RNase inhibitor.
† Store Nuclease-free Water at –20°C, 4°C, or room temp.

C. User-Supplied Required Materials

- *mir*Vana qRT-PCR Primer Set(s) for the miRNA(s) of interest, (see www.ambion.com/miRNA/primers)
- Nuclease-free microcentrifuge tubes and barrier pipet tips (see www.ambion.com/prod/tubes for a complete listing)
- Thermostable DNA polymerase: We recommend using a traditional thermostable (Taq) DNA polymerase, such as SuperTaq™ (P/N AM2050, AM2052). We do not recommend "hot-start" polymerases for use with this kit.
- Thermal cycler for either real-time* or end-point PCR
- For real-time PCR:
  – SYBR® Green I or comparable nucleic acid stain
  – ROX™ normalization dye
  – Optical PCR plates or tubes
- For end-point PCR:
  – PCR plates or tubes (e.g., P/N AM12225, AM12230)
  – Electrophoresis equipment and reagents: We recommend high resolution agarose (e.g., Ambion® Agarose-HR™) in TAE (see: www.ambion.com/prod/electrophoresis)

* This product is compatible with the 5' nuclease detection and dsDNA-binding dye processes covered by patents owned or licensable by Applied Biosystems. No license under these patents is conveyed expressly, by implication, or by estoppel to the purchaser by the purchase of this product. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

D. Related Products Available from Applied Biosystems

*mir*Vana™ miRNA Isolation Kit P/N AM1560	The *mir*Vana miRNA Isolation Kit (patent pending) is designed especially for the isolation of small RNAs, such as microRNA (miRNA), small interfering RNA (siRNA), and small nuclear RNA (snRNA), from tissues and cells. The kit uses a fast and efficient glass fiber filter (GFF) based procedure to isolate total RNA ranging in size from kilobases down to 10-mers. It also includes a procedure to enrich the population of RNAs that are 200 bases and smaller, which enhances the sensitivity of small RNA detection by solution hybridization and Northern blot analysis.
*mir*Vana™ qRT-PCR Primer Set See web or print catalog for P/Ns	The *mir*Vana qRT-PCR Primer Sets, when used in conjunction with the *mir*Vana qRT-PCR miRNA Detection Kit (PN AM1558), enables rapid, simple, and sensitive quantitation of specific miRNAs and other small RNAs in total RNA samples prepared using methods that recover small RNA. Each qRT-PCR Primer Set includes a primer for reverse transcription and a PCR primer pair optimized for sensitive detection of specific miRNAs by qRT-PCR.
SuperTaq™ DNA Polymerase P/N AM2050 and AM2052	SuperTaq DNA Polymerase is a high purity Taq DNA Polymerase originally cloned from *Thermus aquaticus*, and is isolated from an *E. coli* strain that overexpresses this protein. Its heat stability allows DNA polymerization to occur in the 5′ to 3′ direction during repeated heat denaturation and reannealing steps when in the presence of dNTPs, a DNA template and complementary primers. SuperTaq DNA Polymerase can be used in PCR and RT-PCR reactions.

II. *mir*Vana™ qRT-PCR miRNA Detection Procedure

A. Prepare the Reactions and Perform RT-PCR

1. Reagent preparation

WARNING

5X RT PCR Buffer is combustible liquid and vapor. Causes eye, skin, and respiratory tract irritation. May be harmful by inhalation, ingestion, or in contact with skin. Wear appropriate protective eyewear, clothing, and gloves. Use good laboratory techniques when handling.

Dilute *mir*Vana RT primer to 1X concentration

*mir*Vana gene-specific RT Primers are supplied as 10X stock solutions. Dilute to 1X concentration with Nuclease-free Water to make a working stock. Mix thoroughly by vortexing, and centrifuge briefly.

For real-time PCR, add SYBR Green I to the *mir*Vana 5X PCR Buffer

SYBR Green I (not supplied) is sold at 10,000X concentration; we recommend diluting 1 µL of SYBR Green I in 7 µL of dimethyl sulfoxide or Nuclease-free Water. Then add 1.2 µL of the diluted dye to the *mir*Vana 5X PCR Buffer supplied with the kit. Vortex to mix. Note that *mir*Vana 5X PCR Buffer containing SYBR Green I ***can*** be used for traditional end-point PCR analysis.

2. Input RNA Requirements

CAUTION

Human Heart Total RNA is a potential biohazard. Handle as if capable of transmitting infectious disease. If spilled, wash spill site with 10% bleach. Soak up with inert absorbent material (for example; sand, silica gel, acid binder, universal binder, sawdust) and dispose in a proper biohazard location.

RNA must contain the miRNA fraction

Typically, RNA isolation methods that use RNA-binding glass-fiber filters do not quantitatively recover RNA species <200 nt. We recommend using the Ambion® *mir*Vana™ miRNA Isolation Kit (P/N AM1560) or *mir*Vana PARIS™ Kit (P/N AM1556) to purify total RNA or to isolate RNA fractions enriched for small RNA species. Total RNA from other commercial suppliers may or may not contain miRNAs. All of Ambion FirstChoice® Total RNAs from normal human, mouse, and rat tissue are validated for miRNA research. A list is available on our website: www.ambion.com/prod/fcrna

Volume and amount: Use 1 µL containing 10–25 ng RNA

- To get the most reproducible data and to simplify reaction set-up adjust the concentration of RNA samples with Nuclease-free Water so that 1 µL of RNA solution is used per reaction.

- When RNA samples are not limiting, use 10–25 ng of total RNA per reaction. However, relatively abundant miRNAs, such as miR-24, can be detected using as little as 25 pg of the supplied Human Heart Total RNA. The minimum amount of RNA required will depend on the expression level of the miRNA target in the sample.
- 500 ng is the maximum recommended amount of total RNA.

3. Prepare a reverse transcription (RT) Master Mix and mix gently

WARNING

ArrayScript Enzyme Mix may be harmful if inhaled, swallowed, or absorbed through the skin. May cause eye, skin, and respiratory tract irritation. Avoid breathing vapor. Use with adequate ventilation. Avoid contact with eyes and skin. Wear appropriate protective eyewear, clothing, and gloves.

WARNING

5X PCR Buffer Causes eye, skin, and respiratory tract irritation. May be harmful if inhaled, swallowed, or absorbed through the skin. Avoid breathing vapor. Avoid contact with eyes and skin. Wear appropriate protective eyewear, clothing, and gloves.Use good laboratory techniques when handling.

a. Vortex all the RT reagents, except the ArrayScript™ Enzyme Mix just before use. Keep the RT reagents on ice.

b. Prepare an RT Master Mix of the reagents that are shared across most of your samples using Table 1 below.
   - When testing many RNA samples for the presence of one or a few miRNAs, use a ***no RNA Master Mix.*** Prepare a separate Master Mix for each miRNA-specific RT Primer.
   - When testing one or a few RNA samples for many different miRNAs, use a ***no RT Primer Master Mix***. Prepare a separate Master Mix for each experimental RNA sample.
   - Include ~5–10% overage to cover pipetting error.
   - Perform duplicate RT-PCRs for each RNA sample/*mir*Vana qRT-PCR Primer Set pair.
   - For each *mir*Vana qRT-PCR Primer Set in your experiment, include a ***no-template control*** with Nuclease-free Water in place of RNA.

Table 1. Final Composition of the RT Reaction

Per reaction	Component
to 10 µL	Nuclease-free Water
2 µL	*mir*Vana 5X RT Buffer
1 µL	1X *mir*Vana RT Primer*
– µL†	10–25 ng RNA or Nuclease-free Water
0.4 µL	ArrayScript Enzyme Mix
10 µL	final volume

* Diluted from the 10X stock supplied.
† We suggest adjusting RNA concentration so that 1 µL is used.

c. Vortex the RT Master Mix on a low setting to mix.

4. Assemble the RT reaction

a. Aliquot the appropriate volume of RT Master Mix into individual wells of a 96 well PCR plate, or into individual or strip PCR tubes.

b. No RNA Master Mix: Add RNA to each aliquot of RT Master Mix for the experimental samples. For the no-template control reactions, add Nuclease-free Water instead.
No RT Primer Master Mix: Add 1 µL of 1X *mir*Vana RT Primer (diluted from the 10X stock supplied) to each aliquot of RT Master Mix.

c. Mix reactions by pipetting up and down a few times as you add the missing reagent to the RT Master Mix. Briefly centrifuge to collect contents in the wells or tubes.

5. Incubate for 30 min at 37°C, then for 10 min at 95°C

Perform these incubations in a thermal cycler. It is convenient to add a 4°C "hold" step following the 95°C heat inactivation.

a. Incubate 30 min at 37°C for the RT.

b. Incubate 10 min at 95°C to inactivate the ArrayScript.

c. Briefly centrifuge the plate or tubes. Store reactions at –20°C, or place in ice and proceed to the PCR immediately.

6. Assemble PCR Master Mix on ice using Table 2

IMPORTANT

The miRNA detection procedure has been optimized with the supplied mirVana 5X PCR Buffer; it is formulated to minimize the formation of primer-dimers during PCR. Using other PCR buffers may greatly compromise your results.

Thaw the *mir*Vana 5X PCR Buffer at room temperature, not at 37°C. Vortex the *mir*Vana 5X PCR Buffer and *mir*Vana PCR Primers just before use.

Table 2. PCR Master Mix

Per rxn	Component
5 µL	*mir*Vana 5X PCR Buffer (containing SYBR Green I for real-time PCR*)
0.5 µL	50X ROX™ (for real-time PCR only—not supplied)
1 U	Thermostable DNA Polymerase (e.g., 0.2 µL SuperTaq—not supplied)
0.5 µL	*mir*Vana PCR Primers
to 15 µL	Nuclease-free Water

* For instructions, see step II.A.1 on page 5.

7. Gently mix the PCR Master Mix and add 15 µL to each RT rxn

a. Gently vortex the PCR Master Mix and briefly centrifuge to collect it at the bottom of the tube.

b. Set the RT reactions (from step 5) on ice, and add 15 µL of PCR Master Mix to each RT reaction and centrifuge briefly.

8. PCR cycling and analysis

It is ideal to place reactions in a preheated (90–95°C) thermal cycler heat block to start the 95°C denaturation.

Denature:	95°C for 3 min
Cycling:	95°C for 15 sec
	60°C for 30 sec

Real-time PCR

- Continue the PCR for 40 cycles.
- Determine the cycle threshold (C_T) using the software supplied by the thermal cycler manufacturer. We recommend using the automatic baseline determination feature.
- When formatting amplification plots, we recommend setting the Y-axis (Delta Rn) to a log scale ranging from 0.01 to 10.
- Perform dissociation analysis (melt-curve) on the reactions to identify the characteristic peak associated with primer-dimers. Successful PCR amplification of specific miRNA targets should exhibit a single prominent peak that is readily distinguishable from the primer-dimer observed in the no-template control reactions.
- To precisely quantify fold variation in expression of a specific miRNA between experimental samples, amplify the miRNA from a dilution series of the experimental RNA expected to have the highest expression. Then plot the resulting C_T values to generate a standard curve for each *mir*Vana qRT-PCR Primer Set (see Figure 1). The slope of the line is a function of the primer set's amplification efficiency; the equation that represents this line can be used to accurately identify fold-variation between experimental samples.

End-point PCR

- For end-point PCR, it is critical to amplify for an appropriate number of cycles so that 1) the PCR amplification product is readily visible on an agarose gel, and 2) the reaction remains in the exponential phase of amplification. The number of cycles required to meet these two criteria must be empirically determined. As a starting point, we recommend testing 15–25 cycles (see Figure 1).
- Analyze reaction products (10 µL) by electrophoresis on a 3.5% high resolution agarose gel in 1X TAE stained with dye for detecting dsDNA. Gene specific miRNA amplicons should form discrete ~90 bp bands that are easily distinguished from smaller primer-dimer bands that may be seen in the no-template control reaction.
- End-point PCR can be used for qualitative determination of differences in the expression of a given miRNA between two (or more) RNA samples. In addition, a "standard curve" can be constructed to define the approximate magnitude of miRNA expression differences between samples.

III. Troubleshooting

A. Positive Control Reaction

To conduct the positive control reaction, use the 10X hsa-miR-24 RT Primer and the hsa-miR-24 PCR Primers to amplify hsa-miR-24 from 25 ng of the Human Heart Total RNA provided with the kit. Follow the routine protocol described in section I.D starting on page 4. Be sure to include a no-template control.

Expected result

Using end-point PCR, the positive control reaction should yield a single, easily detected, ~90 bp product after 20 cycles of PCR. With real-time PCR analysis, a C_T value of 15–20 is expected (the precise C_T value will depend on the user-defined threshold).

Troubleshooting the positive control reaction

If the positive control reaction does not work as expected, consider the following suggestions:

- Check the reaction set-up carefully.
- It is possible that the thermostable DNA polymerase used is incompatible or inactive; consider trying SuperTaq™ polymerase (P/N AM2050, AM2052).
- RNase contamination could be a problem. Always wipe down pipettors and working surfaces with an RNase decontamination solution such as RNase*Zap*™ Solution (P/N AM9780). Always use tubes and tips that are certified nuclease-free.
- Check that the cycling conditions are set according to these instructions and that the instrument functions properly.

B. Checking your RNA for compatibility with this assay

The hsa-miR-24 qRT-PCR Primer Set provided with the kit can be used to amplify hsa-, mmu-, and rno-miR-24 from RNA derived from human, mouse, or rat, respectively. Our scientists have observed miR-24 in every human tissue tested so far. Therefore, after obtaining a positive result from the positive control reaction described above, you can use the hsa-miR-24 qRT-PCR Primer Set to functionally test whether your RNA is compatible with the *mir*Vana qRT-PCR miRNA Detection assay and contains small RNA species. Another possibility is to use the *mir*Vana qRT-PCR Primer Sets specific for the small RNA species U6 snRNA or 5S rRNA to test your RNA for compatibility.

C. Products in the no-template control reaction

Product from amplification of PCR primer-dimer

We recommend routinely running a no-template control reaction for each *mir*Vana qRT-PCR Primer Set in the experiment. In successful miRNA amplification reactions a single ~90 bp product is synthesized which results in detection of a single product by melt curve analysis on a real-time thermal cycler. Smaller products (end-point) or products with lower T_m (real-time) may be observed in some no-template control reactions; these result from primer-dimer formation. They are typically only detectable late in the cycling protocol (>30 cycles). (Product from primer-dimer can also be amplified in no-RT control reactions.)

Reagent contamination with PCR product

If a product of ~90 bp is observed in the no-template control reaction, there is a good chance that one or more of the PCR reagents, pipettors, or benchtops has been contaminated with PCR products. Unfortunately the only way to remedy contaminated reagent(s) is to replace them.

To avoid PCR contamination, clean the lab bench and the pipettors routinely with a DNA decontamination reagent such as DNA*Zap*™ solution (P/N AM9890). Always use barrier tips to pipette PCR reagents, and store completed PCRs in a different location than the PCR reagents.

D. No RT-PCR product

Perform the positive control reaction

The positive control reaction will identify problems with the *mir*Vana qRT-PCR miRNA Detection Kit reagents, the thermostable DNA polymerase, and the thermal cycler.

Positive control reaction works as expected, but no product from experimental samples

The expression of many miRNAs is developmentally and/or temporally regulated. Therefore, the miRNA may not be expressed in your sample, or its expression may be too low to be detected. You can increase the sensitivity of the assay by using more total RNA—up to 500 ng.

There may be a problem with your RNA sample. Test it for compatibility with this assay by using the hsa-miR-24 qRT-PCR Primer Set to amplify miR-24 as described in section III.B.B on page 10. If your RNA was obtained using a method that includes glass-fiber filter purification, it may not contain the small RNA fraction. We recommend the *mir*Vana miRNA Isolation Kit (P/N AM1560) or *mir*Vana PARIS Kit (P/N AM1556).

IV. Appendix

A. Quality Control

Functional testing

Duplicate real-time PCRs are performed using the supplied hsa-miR-24 qRT-PCR Primer Set and Heart Total RNA sample. A delta-C_t spread of at least 15 cycles is observed between no template control reactions and reactions containing 25 ng of heart RNA.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

Protease testing

Meets or exceeds specification when a sample is incubated with protease substrate and analyzed by fluorescence.

B. Safety Information

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety goggles, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining the MSDS

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com), telephone (650-554-2756; USA), or fax (650-554-2252; USA) your request, specifying the catalog or part number(s) and the name of the product(s). The associated MSDSs will be e-mailed unless you request fax or postal delivery. Requests for postal delivery require 1 to 2 weeks for processing.

Note: For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.