November 2018

RT² PreAMP cDNA Synthesis Handbook

RT² PreAMP cDNA Synthesis Kit
RT² PreAMP Pathway Primer Mix

For synthesis and preamplification of cDNA from small RNA samples and RNA from formalin-fixed, paraffin-embedded samples

Sample to Insight

QIAGEN

Contents

Kit Contents

RT² PreAMP cDNA Synthesis Kit **Catalog no.** **Number of cDNA synthesis reactions**	**(12)** **330451** **12**
Buffer GE	24 µl
5x Buffer BC3	48 µl
cDNA Synthesis Enzyme Mix	12 µl
RNase Inhibitor	12 µl
Control P2	12 µl
RNase-Free Water	1 ml
RT² PreAMP PCR Mastermix	600 µl
Side Reaction Reducer	96 µl
Handbook	1

RT² PreAMP Pathway Primer Mix **Catalog no.** **Number of preamplification reactions**	**(12)** **Varies** **12**
RT² PreAMP Pathway Primer Mix	90 µl

* Each RT² PreAMP Pathway Primer Mix can be used only with the corresponding gene- or pathway-specific RT² Profiler PCR Array. Verify that the lot number of the RT² PreAMP Pathway Primer Mix is compatible with that of the RT² Profiler PCR Array to be used.

Storage

The RT² PreAMP cDNA Synthesis Kit is shipped on dry ice or blue ice packs and must be stored at –20°C upon receipt. To ensure that RT² PreAMP PCR Mastermix does not become contaminated and to avoid repeated freezing and thawing, divide into appropriate aliquots. Store away from any sources of template DNA. RT² PreAMP Pathway Primer Mix is shipped frozen or at ambient temperature. Store at –20°C immediately upon receipt. If entire volume will not be used at the same time, divide into aliquots and store at –20°C. Avoid repeated freezing and thawing.

Under these conditions, these components can be kept for at least 6 months from the date of receipt without any reduction in performance.

Intended Use

The RT² PreAMP cDNA Synthesis Kit and RT² PreAMP Pathway Primer Mix are intended for molecular biology applications. These products are not intended for the diagnosis, prevention or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at

www.qiagen.com/us/support/qa-qc-safety-data/safety-data-sheets/ where you can find, view and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of RT^2 PreAMP cDNA Synthesis Kit and RT^2 PreAMP Pathway Primer Mix is tested against predetermined specifications to ensure consistent product quality.

Introduction

Recent advances in tissue preparation and RNA purification procedures allow researchers to work with very small amounts of RNA such as those from small cell populations, laser capture microdissection samples, fluorescence-activated cell sorting (FACS®) samples, or fine needle aspiration biopsies. However, the low RNA yields obtained from these small samples are often insufficient for reliable gene expression analysis, even using sensitive techniques such as real-time RT-PCR.

Around the world, archives of formalin-fixed, paraffin-embedded (FFPE) tissue sections with known clinical annotations represent a valuable and extensive source of material for biomedical research. However, analysis of RNA from those samples presents many challenges. RNA in FFPE samples is likely to be fragmented and chemically modified by formaldehyde with extensive crosslinking between protein, DNA and RNA. Some of this RNA damage is irreversible and greatly reduces the amount of template available for downstream real-time RT-PCR analysis, thus affecting performance and sensitivity.

The RT^2 PreAMP cDNA Synthesis Kit and RT^2 PreAMP Pathway Primer Mixes are a breakthrough technology enabling expression analysis of hundreds of pathway- or disease-focused genes using as little as 1 ng total RNA from fresh/frozen samples or 100 ng

total RNA from FFPE samples. RT² PreAMP technology uses multiplex, PCR-based preamplification to provide amplification of gene-specific cDNA target templates with minimal bias. The RT² PreAMP cDNA Synthesis Kit and RT² PreAMP Pathway Primer Mixes enable synthesis and preamplification of cDNA from total RNA samples, prior to gene expression analysis using RT² Profiler PCR Arrays. Each RNA sample can be used to prepare enough cDNA for gene expression analysis of up to 4 different pathways.

Principle and Procedure

The RT² PreAMP cDNA Synthesis Kit and RT² PreAMP Pathway Primer Mixes provide a convenient and quick workflow, accessible for routine use in every research laboratory. The procedure consists of 2 steps: cDNA synthesis and preamplification.

cDNA synthesis

The RT² PreAMP cDNA Synthesis Kit provides sufficient reagents for synthesis of cDNA from 12 RNA samples. The kit includes a built-in external RNA control template that is detected by the reverse transcription control (RTC) in RT² Profiler PCR Arrays. This control allows detection of any inhibitors of reverse transcription that could compromise the efficiency of cDNA synthesis reactions.

Preamplification of cDNA for pathway-specific genes

Each RT² PreAMP Pathway Primer Mix is specific for one RT² Profiler PCR Array. During the amplification step, the RT² PreAMP Pathway Primer Mix enables amplification of cDNA specific for the genes targeted by the RT² Profiler PCR Array. A cDNA synthesis reaction from 1-100 ng total RNA from fresh/frozen samples or 100 ng–1 µg total RNA from FFPE samples provides sufficient cDNA for amplification by 4 different RT² PreAMP Pathway Primer Mixes, allowing gene expression analysis of up to 4 different pathways. Following preamplification, the Side

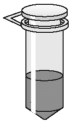
Reaction Reducer eliminates residual primers. Preamplified cDNA is then ready for PCR array analysis using the appropriate RT^2 Profiler PCR Array.

Gene expression analysis using RT^2 Profiler PCR Arrays

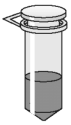
For PCR array analysis, preamplified cDNA is mixed with RT^2 SYBR® Green Mastermix. Various RT^2 SYBR Green Mastermixes are available for use with different real-time cyclers. The mixture is aliquoted into the wells of an RT^2 Profiler PCR Array, which contain predispensed gene-specific primer pairs. PCR is performed, and relative expression is determined using the $\Delta\Delta C_T$ method.

RT² PreAMP cDNA Synthesis Procedure

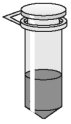
Purified RNA



Genomic DNA elimination and cDNA synthesis

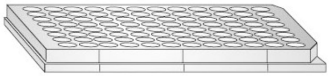


Multiplex PCR



Add Side Reaction Reducer

Aliquot mix into RT² Profiler PCR Arrays



Cycle in real-time cycler

Analyze results

Description of protocols

This handbook contains 3 protocols. The first protocol describes first-strand cDNA synthesis from your RNA samples (page 18). After completion of this protocol, cDNA samples are preamplified in the second protocol (page 20). After preamplification, samples are ready to be used for analysis with an RT² Profiler PCR Array. For RT² Profiler PCR Array analysis, follow the instructions in the third protocol (page 22) and in the *RT² Profiler PCR Array Handbook*.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

In addition to the RT² PreAMP cDNA Synthesis Kit and RT² PreAMP Pathway Primer Mix, the following are required:

- Purified RNA samples (for recommended purification methods, see page 14)
- **RT² Profiler PCR Array**
  RT² Profiler PCR Arrays are available in different formats, to suit different real-time cyclers. For full details, see the *RT² Profiler PCR Array Handbook*.
  Each RT² PreAMP Pathway Primer Mix is specific for a particular pathway or disease RT² Profiler PCR Array. Check the label to verify that the correct pathway-specific RT² PreAMP Pathway Primer Mix is used for the RT² Profiler PCR Array. Verify that the lot number of the RT² PreAMP Pathway Primer Mix is compatible with that of the RT² Profiler PCR Array to be used. We strongly recommend that RT² PreAMP Pathway Primer Mix and the RT² Profiler PCR Array are purchased together whenever possible. If the RT² PreAMP Pathway Primer Mix and the RT² Profiler PCR Array have been purchased at different times, check with QIAGEN Technical Services to ensure their compatibility.
- RT² SYBR Green Mastermix suitable for use with your real-time cycler. RT² SYBR Green Mastermixes available include the following:
  - RT² SYBR Green qPCR Mastermix: suitable for use with real-time cyclers that do not require a reference dye, including Bio-Rad® models CFX96™ and CFX384™, Bio-Rad/MJ Research models Chromo4™ and DNA Engine Opticon® 2, and Roche® LightCycler® 480 (96-well and 384-well)
  - RT² SYBR Green Fluor qPCR Mastermix: suitable for use with the following real-time cyclers: Bio-Rad models iCycler®, iQ™5, MyiQ™, MyiQ2

- RT² SYBR Green ROX™ qPCR Mastermix: suitable for use with the following real-time cyclers: Applied Biosystems® models 5700, 7000, 7300, 7500 (Standard and Fast), 7700, 7900HT (Standard and Fast 96-well block, 384-well block), StepOnePlus™, ViiA™ 7 (Standard and Fast 96-well block, 384-well block); Eppendorf® Mastercycler® ep realplex models 2, 2S, 4, 4S; Stratagene® models Mx3000P®, Mx3005P®, Mx4000®; Takara TP-800
- RT² SYBR Green ROX FAST Mastermix: suitable for use with the Rotor-Gene® Q and other Rotor-Gene cyclers

- Real-time PCR cycler with 0.2 ml tube heat block, heated lid, and 10–100 µl reaction capacity
- 0.2 ml tubes or 8-tube strip PCR tubes with caps
- Multichannel pipettor
- Nuclease-free pipet tips and tubes
- Optional: XpressRef Universal Total RNA to control PCR conditions is available for human (cat. no. 338112), mouse (cat. no. 338114) and rat (cat. no. 338116)

Important Notes

Preparing a workspace free of DNA contamination

For accurate and reproducible PCR array results, it is important to avoid contamination of the assay with foreign DNA. Any DNA contamination will artificially inflate the SYBR Green signal during real-time RT-PCR, yielding skewed gene expression profiles and false-positive signals. The most common sources of DNA contamination are the products of previous experiments spread into the air of the working environment. To set up and maintain a working environment free of DNA contamination, follow the guidelines below.

- Wear gloves throughout the procedure. Use only fresh PCR-grade reagents (water) and labware (tips and tubes).
- Physically separate the workspaces used for PCR setup and post-PCR processing or non-PCR operations. Decontaminate the PCR workspace and labware (pipettor barrels, tube racks, etc.) before each use with UV light (to render any contaminating DNA ineffective in PCR through the formation of thymidine dimers) or with 10% bleach (to chemically inactivate and degrade any DNA).
- Close all tubes containing PCR products once you are finished adding or removing volumes. Before discarding any labware (tips or tubes) containing PCR products or other DNA, treat with 10% bleach.
- Do not remove the RT^2 Profiler PCR Array from its protective sealed bag until immediately before use. Do not leave labware (tubes and tip boxes) exposed to the air for long periods of time.
- Do not open any previously run and stored RT^2 Profiler PCR Array. Removing the thin-wall 8-cap strips or the adhesive film from PCR arrays releases PCR product DNA into the air where it may affect the results of future real-time PCR experiments.

RNA preparation, quantification, and quality control

The most important prerequisite for any gene expression analysis experiment is purification of consistently high-quality RNA from every experimental sample. Residual traces of proteins, salts, or other contaminants may degrade the RNA or decrease the efficiency of enzyme activities necessary for optimal reverse transcription and real-time PCR performance.

Laser capture microdissected (LCM) samples

We recommend capturing LCM samples on Arcturus® CapSure® Caps and purifying RNA using the Arcturus PicoPure® RNA Isolation Kit following the manufacturer's instructions. Special care should be taken during sample preparation and microdissection to minimize RNA degradation. For example, LCM sessions longer than 30 minutes per slide may lead to precipitous loss in recovery of intact RNA. Keep the staining and dissection procedure as short as possible.

Fine needle aspiration biopsies (FNAB) and other small biological samples

We recommend use of the RNeasy® Micro Kit (cat. no. 74004) for most small samples with a small number of cells such as fine needle aspiration biopsies, manual dissection samples, or FACS samples. In addition, the Arcturus PicoPure RNA Isolation Kit can also be used for non-LCM samples such as cell samples in suspension. Irrespective of the kit used, it is important to perform DNase treatment during the procedure.

FFPE samples

We recommend use of the RNeasy FFPE Kit (cat. no. 73504) for RNA purification from FFPE samples.

Total RNA isolated using a phenol-based method

Total RNA from any biological source material prepared using a phenol-based method (e.g., QIAzol® Lysis Reagent, TRIzol® Reagent, RNAzol® Reagent) should be further purified using

the RNeasy Mini Kit (cat. no. 74104). It is important to perform the on-column DNase digestion step described in the *RNeasy Mini Handbook*.

RNA quantification and quality control

For optimal performance of RT² PreAMP procedures and best results from the RT² Profiler PCR Array, all RNA samples should be resuspended in RNase-free water. Do not use DEPC-treated water for resuspension.

Concentration and purity determined by UV spectrophotometry

The concentration and purity of RNA should be determined by measuring the absorbance in a spectrophotometer. Prepare dilutions and measure absorbance in 10 mM Tris·Cl,*pH 8.0. The spectral properties of nucleic acids are highly dependent on pH. An absorbance reading of 1.0 at 260 nm in a 1 cm detection path corresponds to an RNA concentration of 40 µg/ml.

- A_{260}:A_{230} ratio should be greater than 1.7
- A_{260}:A_{280} ratio should be 1.8 to 2.0
- Concentration determined by A_{260} should be >40 µg/ml

Integrity of RNA from fresh/frozen samples

If RNA samples are at least 2 ng, quality control can be performed by analyzing 200 pg of each sample on an Agilent® Bioanalyzer using an RNA 6000 Pico LabChip®. Verify that there is a sharp distinction at the small side of both the 18S and 28S ribosomal RNA (rRNA) peaks. Any smearing or shoulder on the rRNA peaks indicates that degradation has occurred in the RNA sample.

* When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Due to the extremely low yield of RNA from small samples, it is often impossible to confirm the quality of total RNA using any analytical means. Therefore, it is essential to choose a suitable RNA purification method for the biological sample as described above and follow the manufacturer's protocol carefully.

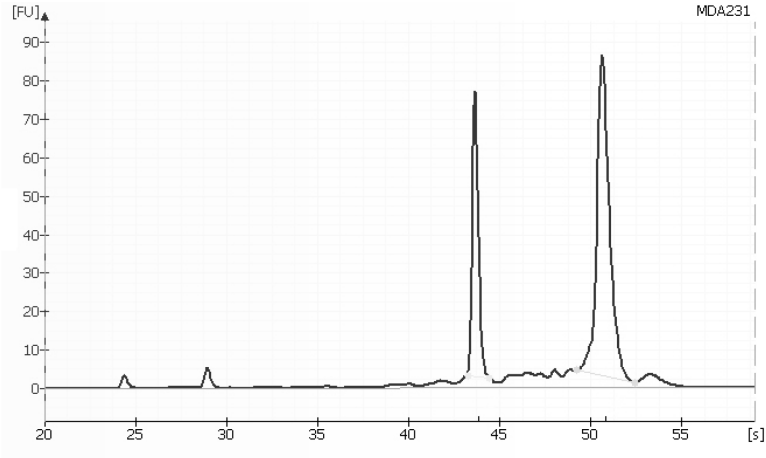


Figure 1. Ribosomal RNA band integrity is important for optimal PCR array results. An Agilent Bioanalyzer electropherogram of a high-quality total RNA preparation shows sharp peaks without shoulders (especially to the left of each peak) for the 18S (left) and 28S (right) ribosomal RNA.

Integrity of RNA from FFPE samples

RNA from FFPE tissue samples is expected to show some degree of degradation. Run an aliquot of each RNA sample on a denaturing agarose gel, or alternatively, characterize the samples using an RNA 6000 Nano Chip on an Agilent Bioanalyzer. Assess the extent of RNA degradation. The usual length of FFPE RNA fragments is expected to be 100–1000 bases.

Genomic DNA contamination

Eliminating genomic DNA contamination is essential for obtaining optimal real-time gene expression profiling results using RT^2 Profiler PCR Arrays. The genomic DNA control in each

RT² Profiler PCR Array specifically tests for genomic DNA contamination in each sample during each run. A genomic DNA control threshold cycle value of less than 30 after preamplification indicates the presence of a detectable amount of genomic DNA contamination that should be addressed.

To remove any residual contamination from your RNA samples, we recommend RNA purification using the RNeasy Mini Kit, including the optional on-column DNase digestion step or using the RNeasy FFPE Kit.

Starting RNA amounts

Quantifying total RNA from very small samples using UV spectrophotometry is often impossible. Instead, estimate the total RNA amount by assuming that each eukaryotic cell contains an average of ~10 pg of total RNA or less. Note that the yield of RNA varies widely from cell type to cell type.

The RT² PreAMP cDNA Synthesis Kit and RT² PreAMP Pathway Primer Mix generate sufficient template for gene expression analysis using RT² Profiler PCR Arrays with as little as 1–100 ng total RNA (from fresh/frozen samples) or 100 ng–1 µg total RNA (from FFPE samples) input into each first-strand cDNA synthesis reaction prior to preamplification. Each cDNA synthesis reaction allows the user to perform as many as 4 preamplification reactions and 4 RT² Profiler PCR Array analyses. However, the optimal amount of starting material depends on the relative abundance of the transcripts of interest. Lower abundance transcripts require more RNA; higher abundance transcripts require less RNA. Greater amounts of input total RNA yield a greater number of positive calls; that is, genes expressed in the linear dynamic range of the method. Lower amounts of input total RNA yield a smaller number of positive calls and increase false negative calls.

The RT² PreAMP cDNA Synthesis Kit and RT² PreAMP Pathway Primer Mix maximize the number of positive calls at low amounts of total RNA. For successful results and maximum positive call rates, we recommend that first-time users start by using 10–50 ng total RNA from

fresh/frozen samples or 500 ng–1 µg total RNA from FFPE samples. It is also important to use a consistent amount of total RNA for all samples to be characterized and compared in a single experiment.

Protocol: First-Strand cDNA Synthesis

Important points before starting

- RNA samples are very sensitive to RNase digestion; therefore, wear gloves and maintain an RNase-free work area while performing this protocol.
- The RT² PreAMP cDNA Synthesis Kit is not compatible with the chemicals in DNA-free™ kits from Ambion. If your RNA sample has been treated with DNA-free reagents, contact QIAGEN Technical Services.

Procedure

1. Prepare the genomic DNA elimination mix for each RNA sample in a sterile PCR tube according to Table 1. Mix gently by pipetting up and down and then centrifuge briefly.

Table 1. Genomic DNA elimination mix

Component	Amount for 1 sample (RNA from fresh/frozen sample)	Amount for 1 sample (RNA from FFPE sample)
RNA	1 ng–100 ng	100 ng–1 µg
Buffer GE	2 µl	2 µl
RNase-free water	Variable	Variable
Total volume	**10 µl**	**10 µl**

2. Incubate the genomic DNA elimination mix at 42°C for 5 minutes, then place immediately on ice for at least 1 minutes.
3. Prepare the reverse-transcription mix according to Table 2. For multiple reactions, scale up the volumes shown in Table 2 accordingly.

Table 2. Reverse-transcription mix

Component	Volume for 1 reaction
5x Buffer BC3	4 µl
Control P2	1 µl
cDNA Synthesis Enzyme Mix	1 µl
RNase Inhibitor	1 µl
RNase-free water	3 µl
Total volume	**10 µl**

4. Add 10 µl reverse-transcription mix to each tube containing 10 µl genomic DNA elimination mix. Mix gently by pipetting up and down. Centrifuge briefly to remove any air bubbles and collect all the liquid to the bottom of the tube.
5. Incubate at 42°C for exactly 30 minutes. Then immediately stop the reaction by incubating at 95°C for 5 minutes.
6. Place the reactions on ice and proceed with the preamplification protocol.

   If you wish to store the reactions overnight prior to real-time PCR, transfer to a -20°C freezer. Longer storage times are not recommended.

Protocol: Preamplification of cDNA Target Templates

Important points before starting

- Each RT² PreAMP Pathway Primer Mix is specific for a particular pathway or disease RT² Profiler PCR Array. Check the label to verify that the correct pathway-specific RT² PreAMP Pathway Primer Mix is used for the RT² Profiler PCR Array. Verify that the lot number of the RT² PreAMP Pathway Primer Mix is compatible with that of the RT² Profiler PCR Array to be used. If the RT² PreAMP Pathway Primer Mix and the RT² Profiler PCR Array have been purchased at different times, check with QIAGEN Technical Services to ensure their compatibility.

Procedure

1. Thaw the RT² PreAMP PCR Mastermix and RT² PreAMP Pathway Primer Mix at room temperature. If precipitates are visible, warm the reagents at 42°C for 1 minute and vortex briefly to dissolve. Repeat if necessary.
2. Prepare the preamplification mix according to Table 3. For multiple reactions, scale up the volumes shown in Table 3 accordingly.

Table 3. Preamplification mix

Component	Amount for 1 sample
RT² PreAMP PCR Mastermix	12.5 µl
RT² PreAMP Pathway Primer Mix	7.5 µl
Total volume	**20 µl**

3. Pipet 5 µl first-strand cDNA synthesis reaction (from step 6, page 21) into a 0.2-ml PCR tube, then add 20 µl preamplification mix.

4. Mix gently by pipetting up and down. Spin briefly to remove any air bubbles and collect all the liquid to the bottom of the tube.
5. Program the real-time cycler according to Table 4 or Table 5. Place the tubes in the real-time cycler and start the program.

Table 4. Cycling conditions for preamplification of cDNA from fresh/frozen samples

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA *Taq* Polymerase is activated by this heating step.
12	15 s 2 min	95°C 60°C	
Hold		4°C	

Table 5. Cycling conditions for preamplification of cDNA from FFPE samples

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA *Taq* Polymerase is activated by this heating step.
8	15 s 2 min	95°C 60°C	
Hold		4°C	

6. When cycling is finished, take the tubes from the real-time cycler and place on ice.
7. Add 2 µl Side Reaction Reducer to each preamplified reaction. Mix gently by pipetting up and down. Spin the tubes briefly to remove any air bubbles and collect all the liquid at the bottom of the tube.
8. Incubate at 37°C for 15 minutes followed by heat inactivation at 95°C for 5 minutes.
9. Immediately add 84 µl nuclease-free water. Mix well.
10. Place on ice prior to real-time PCR, or store overnight at –20°C.

Protocol: Real-Time PCR Using RT² Profiler PCR Arrays

Important points before starting

- Ensure that the RT² SYBR Green Mastermix and the RT² Profiler PCR Array format are suitable for your real-time cycler. An incorrect RT² Profiler PCR Array format will not fit the real-time cycler properly and may damage the real-time cycler. For more details, see the *RT² Profiler PCR Array Handbook*.
- For accuracy and precision, ensure that micropipettors are calibrated before beginning the protocol. Be sure not to introduce bubbles into the wells of the RT² Profiler PCR Array when pipetting.

Procedure

1. Briefly centrifuge the RT² SYBR Green Mastermix (10–15 s) to bring the contents to the bottom of the tube.

   Note: As the RT² SYBR Green Mastermix contains HotStart DNA *Taq* Polymerase that is active only after heat activation, reactions can be prepared at room temperature (15–25°C).

2. Prepare the PCR components mix in a 5 ml tube or in a loading reservoir depending on the RT² Profiler PCR Array format, as described in Table 6.

Table 6. PCR components mix

Array format	96-well A, C, D, F	384-well E, G	Rotor-Disc® 100 R
2x RT² SYBR Green Mastermix	1275 µl	550 µl	1150 µl
Preamplification reaction (from step 10, page 21)	102 µl	102 µl	102 µl
RNase-free water	1173 µl	448 µl	1048 µl
Total volume	**2550 µl**	**1100 µl**	**2300 µl**

Note: This provides an excess volume of ~140–300 µl to allow for pipetting errors. Perform pipetting steps as precisely as possible to ensure that each well receives the required volume.

3. Continue from the corresponding step 3 onwards of the "Real-Time PCR for RT² Profiler PCR Arrays" Protocol in the *RT² Profiler PCR Array Handbook*.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: **www.qiagen.com/us/shop/pcr/real-time-pcr-enzymes-and-kits/two-step-qrt-pcr/rt2-preamp-cdna-synthesis-kit/#resources**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit **www.qiagen.com**).

	Comments and suggestions
High C_T values for several genes including housekeeping genes	
a) RNA input was less than recommended lower limit of 1 ng	Increase amount of input RNA.
b) Poor quality RNA	Check the concentration and quality of RNA samples as described in "Important Notes" (page 13).
Genomic DNA contamination	
a) DNase treatment not performed	Perform DNase treatment during RNA purification. If purifying RNA using the RNeasy Mini Kit, perform the DNase treatment step. If genomic DNA contamination is difficult to remove, fold-changes in gene expression may still be obtained. However, it will then be very important to validate any results for individual genes by a separate more rigorous real-time PCR analysis that includes a "minus RT" control.
b) DNA contamination of reagents or labware	Follow the guidelines for preparing a workspace free of DNA contamination described in "Important Notes" (page 13).
Poor reverse transcription efficiency	
Poor quality RNA	If possible, recheck the A_{260}:A_{280} and A_{260}:A_{230} ratios of RNA samples in RNase-free Tris pH 8.0 buffer. If necessary, repurify RNA samples with a spin-column based clean up method, such as the RNeasy Mini Kit.

		Comments and suggestions
Poor PCR amplification efficiency		
a)	Sensitivity of real-time cycler	Different real-time cyclers have different levels of sensitivity. If an average C_T^{PPC} value of 20 ± 2 is difficult to obtain for your cycler, the observed average C_T^{PPC} value should be acceptable as long as it does not vary by more than 2 cycles between PCR arrays being compared.
b)	Incorrect cycling parameters	Ensure that the initial heat activation step at 95°C was lengthened to 10 minutes from the shorter time in the default program. Ensure that all other cycle parameters were correctly entered according to the recommendations in the *RT² Profiler PCR Array Handbook*.
c)	Poor quality RNA	If possible, recheck the A_{260}:A_{280} and A_{260}:A_{230} ratios of RNA samples in RNase-free Tris pH 8.0 buffer. If necessary, repurify RNA samples with a spin-column based clean up method, such as the RNeasy Mini Kit.

Appendix A: Data Analysis

Please refer to the "Real-Time PCR for RT^2 Profiler PCR Arrays" Protocol in the RT^2 Profiler PCR Array Handbook for detailed information on data analysis. Experimental C_T values for all wells can be exported to a blank Excel® spreadsheet. Data analysis can then be conducted at QIAGEN'S GeneGlobe Data Analysis Center using a software-based tool or with a spreadsheet-based tool that can be downloaded from a QIAGEN website.

Note: The GeneGlobe Data Analysis Center is a web resource for the analysis of real-time PCR or NGS data (**www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/?akamai-feo=off**). To access the center, new users can register online. Once on the site, the data analysis software can be found under "Analysis." The RT^2 Profiler PCR Array Data Analysis spreadsheet can be found under "Product Resources/Performance Data" at **www.qiagen.com/us/shop/pcr/primer-sets/rt2-profiler-pcr-arrays/#resources**.

Appendix B: Preamplification Prior to Real-Time PCR Using RT² qPCR Primer Assays

This protocol is for preamplification and real-time PCR when using RT² qPCR Primer Assays for low-throughput gene expression analysis of up to 10 genes instead of high-throughput RT² Profiler PCR Arrays. In this protocol, RT² qPCR Primer Assays are used for preamplification instead of an RT² PreAMP Pathway Primer Mix.

Procedure

1. Perform cDNA synthesis as described in the protocol on page 18-19.
2. Dilute RT² qPCR Primer Assay(s) for up to 10 genes of interest as described in Table 7.

Table 7. RT² qPCR Primer Assay mix

Number of genes for expression analysis	RT² qPCR Primer Assay (10 µM)	Nuclease-free water
1 gene	1 assay x 40 µl = 40 µl	960 µl
2 genes	2 assays x 40 µl each = 80 µl	920 µl
3 genes	3 assays x 40 µl each = 120 µl	880 µl
4 genes	4 assays x 40 µl each = 160 µl	840 µl
5 genes	5 assays x 40 µl each = 200 µl	800 µl
6 genes	6 assays x 40 µl each = 240 µl	760 µl
7 genes	7 assays x 40 µl each = 280 µl	720 µl
8 genes	8 assays x 40 µl each = 320 µl	680 µl
9 genes	9 assays x 40 µl each = 360 µl	640 µl
10 genes	10 assays x 40 µl each = 400 µl	600 µl

3. Thaw the RT² PreAMP PCR Mastermix at room temperature. If precipitates are visible, warm the reagents at 42°C for 1 minute and vortex briefly to dissolve. Repeat if necessary.

4. Prepare preamplification mix according to Table 8.

   For multiple reactions, scale up the volumes shown in Table 8 accordingly.

Table 8. Preamplification mix

Component	Amount for 1 sample
RT² PreAMP PCR Mastermix	12.5 µl
RT² qPCR Primer Assay mix	7.5 µl
Total volume	**20 µl**

5. Pipet 5 µl single-strand cDNA synthesis reaction (from step 6, page 19) into a 0.2 ml PCR tube. Then add 20 µl preamplification mix.
6. Mix gently by pipetting up and down. Spin briefly to remove any air bubbles and collect all the liquid to the bottom of the tube.
7. Program the real-time cycler according to Table 9. Place the tubes in the real-time cycler and start the program.

Table 9. Cycling conditions for preamplification

Cycles	Duration	Temperature	Comments
1	10 min	95°C	HotStart DNA *Taq* Polymerase is activated by this heating step.
8	15 s 2 min	95°C 60°C	
Hold		4°C	

8. When cycling is finished, take the tubes from the real-time cycler and place on ice.
9. Add 2 µl Side Reaction Reducer to each preamplified reaction. Mix gently by pipetting up and down. Spin the tubes briefly to remove any air bubbles and collect all the liquid at the bottom of the tube.
10. Incubate at 37°C for 15 minute followed by heat inactivation at 95°C for 5 minutes.
11. Immediately add 28 µl nuclease-free water. Mix well.

12. Place on ice prior to real-time PCR, or store overnight at –20°C.
13. Perform real-time RT-PCR using RT² qPCR Primer Assays as described in the *RT² qPCR Primer Assay Handbook* using 1 µl preamplification reaction as template.

Appendix C: Bench Protocol for First-Strand cDNA Synthesis, Preamplification, and Real-Time PCR

Note: Before using this bench protocol, you should be completely familiar with the safety information and detailed protocols in this handbook and the *RT² Profiler PCR Array Handbook*.

Important point before starting

- Ensure that the pathway and lot number of the RT² PreAMP Pathway Primer Mix are compatible with those of the RT² Profiler PCR Array.

Procedure

1. Add 2 µl Buffer GE to 8 µl RNA (1–100 ng for RNA from fresh/frozen sample; 100 ng-1 µg for RNA from FFPE sample). Incubate at 42°C for 5 minutes and immediately chill on ice.
2. Prepare an RT mix as shown below.

Component	Volume for 1 reaction
5x Buffer BC3	4 µl
Control P2	1 µl
cDNA Synthesis Enzyme Mix	1 µl
RNase Inhibitor	1 µl
RNase-free water	3 µl

3. Add 10 µl RT mix to 10 µl Buffer GE–RNA from step 1. Incubate at 42°C for 30 minutes and heat at 95°C for 5 minutes. Place on ice or store at –20°C until use.
4. Mix the following components in a PCR tube.

Component	Amount for 1 sample
RT² PreAMP PCR Mastermix	12.5 µl
RT² PreAMP Pathway Primer Mix	7.5 µl
cDNA from step C3	5 µl

5. For cDNA from fresh/frozen samples, cycle in a real-time cycler as follows: 95°C, 10 minutes; 12 cycles of 95°C for 15 seconds, 60°C for 2 minutes; 4°C hold. For cDNA from FFPE samples, cycle in a real-time cycler as follows: 95°C, 10 minutes; 8 cycles of 95°C for 15 sec, 60°C for 2 minutes; 4°C hold.
6. Add 2 µl Side Reaction Reducer, incubate at 37°C for 15 minutes followed by heat inactivation at 95°C for 5 minutes.
7. Add 84 µl nuclease-free water. Place on ice or store at –20°C until use.
8. Prepare the following mix for use with a 96-well RT² Profiler PCR Array.

Component	Amount for 1 sample
2x RT² SYBR Green Mastermix	1275 µl
Preamplification reaction from step C7	102 µl
RNase-free water	1173 µl

9. Add 25 µl of the mix to each well of the PCR array. Cycle as follows: 95°C, 10 minutes; 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds.
10. Program the cycler to detect and record the SYBR Green signal during the annealing step of each cycle.

Ordering Information

Product	Contents	Cat. no.
RT² PreAMP cDNA Synthesis Kit (12)	For 12 x 20 µl first strand cDNA synthesis reactions: Buffer GE, 5x Buffer BC3, cDNA Synthesis Enzyme Mix, RNase Inhibitor, Control P2, RNase-Free Water; for 48 x 25 µl preamplification reactions: RT² PreAMP PCR Mastermix (600 µl); Side Reaction Reducer (96 µl)	330451
RT² PreAMP Pathway Primer Mix	For 12 preamplification reactions: 90 µl primer mix for a specific RT² Profiler PCR Array	Varies
RT² Profiler PCR Array	Arrays of assays for disease, pathway, or functionally related genes; available in 96-well, 384-well, and Rotor-Disc 100 format	Varies
RT² SYBR Green qPCR Mastermix (2)*	For 2 x 96 assays in 96-well plates; suitable for use with real-time cyclers that do not require a reference dye; 2 x 1.25 ml mastermix	330500
RT² SYBR Green Fluor qPCR Mastermix (2)*	For 2 x 96 assays in 96-well plates; suitable for use with real-time cyclers that use fluorescein reference dye; 2 x 1.25 ml mastermix	330510
RT² SYBR Green ROX qPCR Mastermix (2)*	For 2 x 96 assays in 96-well plates; suitable for use with real-time cyclers that use ROX reference dye; 2 x 1.25 ml mastermix	330520

* Larger kit sizes available; please inquire.

RT² SYBR Green ROX FAST Mastermix (2)*	For 2 x 96 assays in 96-well plates; suitable for use with real-time cyclers that use ROX reference dye, including the Rotor-Gene Q and Rotor-Gene 6000; 2 x 1.25 ml mastermix	330620

Related products

Human XpressRef Universal Total RNA	2 tubes each containing 100 µg human RNA at 1 mg/ml	338112
Mouse XpressRef Universal Total RNA	2 tubes each containing 100 µg mouse RNA at 1 mg/ml	338114
Rat XpressRef Universal Total RNA	2 tubes each containing 100 µg rat RNA at 1 mg/ml	338116
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free reagents and buffers	74104
RNeasy Micro Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free DNase I, Carrier RNA, RNase-free reagents and buffers	74004
RNeasy FFPE Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes, Proteinase K, RNase-Free DNase I, DNase Booster Buffer, RNase-free buffers, RNase-Free Water	73504
PAXgene Blood RNA Kit (50)	50 PAXgene Spin Columns, 50 PAXgene Shredder Spin Columns, Processing Tubes, RNase-Free DNase I, RNase-free reagents and buffers. To be used in conjunction with PAXgene Blood RNA Tubes	762174
RT² PCR Array Loading Reservoir	12 x 5 ml capacity, irradiation-sterilized loading reservoirs	338162

* Larger kit sizes available; please inquire.

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Document Revision History	
R1	Updated template.
03/2011	Updated data analysis procedure.

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11/2018 HB-0502-002

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