November 2018

# RT² HT First Strand Handbook

## For cDNA synthesis from 96 samples

Sample to Insight

QIAGEN

# Contents

# Kit Contents

| **RT² HT First Strand Kit**<br>**Catalog no.**<br>**Number of reverse-transcription reactions** | **(96)**<br>**330411**<br>**96** |
|---|---|
| Tube of Buffer GE2 (gDNA elimination buffer): 750 µl | 1 |
| 5 ml Reagent Reservoir | 2 |
| Tube of BC4 Reverse Transcriptase Mix: 750 µl | 1 |
| Blank 96-Well Plate | 1 |
| Foil Adhesive Sealing Film | 2 |
| Compression Mat | 1 |

# Storage

The Buffer GE2 and BC4 Reverse Transcriptase Mix tubes included in this kit are shipped on dry ice and must be stored at -20°C upon receipt. When stored properly at -20°C, their quality is guaranteed for 6 months.

The foil sealing films, 96-well plate, compression mat, and reagent reservoir can be stored at room temperature.

All reagents are stable for 6 months after receipt if stored at the recommended temperature.

# Intended Use

The RT² HT First Strand Kit is intended for molecular biology applications. This product is 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$^2$ HT First Strand Kit is tested against predetermined specifications to ensure consistent product quality.

# Introduction

Real-time reverse transcription (RT) PCR is the most sensitive and reliable method for gene expression analysis currently available. Its wide dynamic range makes real-time RT-PCR the preferred choice for the simultaneous quantification of both rare and abundant genes in the same sample. For successful RT-PCR, starting RNA samples have to be converted to the first strand cDNA, which is the template for real-time PCR. However, researchers often face the challenge of eliminating genomic DNA contamination in their RNA samples and burden of carrying out reverse transcriptions for a large number of the RNA samples.

The RT² HT First Strand Kit provides a rapid and convenient procedure for efficient first strand cDNA synthesis and genomic DNA elimination of your RNA samples. Its unique format enables easy parallel processing of 96 RNA samples for reverse transcription at the same time. The synthesized cDNA is ready to use in real-time PCR for monitoring expression of multiple genes when used with RT² SYBR® Green qPCR Mastermixes, RT² qPCR Primer Assays, FlexiPlate siRNA, or with other experimental procedures requiring cDNA from a large number of samples.

**Benefits of the RT² HT First Strand Kit:**

- **Easy and fast**: Everything is included in 1 kit to eliminate genomic DNA and complete first strand cDNA synthesis for 96 RNA samples simultaneously.
- **Eliminate false positives**: Optimized DNA removal buffer prevents false positive signals due to amplification of genomic DNA contamination.
- **Ensure RT efficiency and consistency**: Built-in external RNA control verifies lack of enzyme inhibitors and efficient reverse transcription

# Principle and Procedure

The RT² HT First Strand Kit procedure comprises 2 steps: elimination of genomic DNA contamination, and reverse transcription, which enable fast and easy handling of 96 RNA samples simultaneously.

**Eliminate Genomic DNA Contamination**

A proprietary genomic DNA elimination buffer efficiently removes any residual genomic DNA from your RNA sample. Then, the optimized formulation also allows you to directly use the RNA preparation for reverse transcription and finally real-time PCR without affecting reaction

performance. By eliminating genomic DNA contamination, real-time PCR signal intensities accurately reflect the relative level of gene-specific mRNA transcript.

**Reverse Transcription**

After genomic DNA elimination, the RNA sample is ready for reverse transcription using a RT master mix, which includes every component for first strand cDNA synthesis. Random hexamers and oligo-dT prime reverse transcription in an unbiased manner and capture more difficult-to-detect genes. The MMLV reverse transcriptase, optimized magnesium concentration and other buffer components maximize cDNA product yield.

**Control**

A built-in external RNA control helps test for inhibitors of reverse transcription. A pair of specific primers (RTC) detect template synthesized from this built-in external RNA control. A reproducible threshold cycle value from this control across your samples indicates a consistent high level of RNA quality and transcription efficiency giving you a greater degree of confidence in your results.

# 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.

## For RNA Isolation

- SV 96 Total RNA Isolation System (Promega, catalog no. Z3500 or Z3505) for 96 samples, or
- RNeasy® Mini Kit (cat. no. 74104) for individual samples

## For First Strand cDNA Synthesis

- Multi-channel pipette
- Centrifuge
- Plate centrifuge
- Standard thermal cycler or water bath

## For Real-time PCR

### RT² SYBR Green qPCR Mastermix

Be sure to pick the correct one for the instrumentation in your laboratory.

**RT² SYBR Green ROX™ qPCR Mastermix**

Specifically designed for all ABI and Stratagene® Instrumentation and Eppendorf® Mastercycler® ep *realplex* Instruments with ROX filter set.

| Catalog no. | Size |
|---|---|
| 330520 | For 200 25-µl reactions (2.5 ml) |
| 330529 | For 2000 25-µl reactions (25 ml) |

### RT² SYBR Green Fluor qPCR Mastermix

Specifically designed for BioRad iCycler®, MyiQ™, and iQ™5 Instrumentation.

| Catalog no. | Size |
|---|---|
| 330510 | For 200 25-µl reactions (2.5 ml) |
| 330519 | For 2000 25-µl reactions (25 ml) |

### RT² SYBR Green qPCR Mastermix

Specifically designed for instrumentation that does not require a reference dye: BioRad® (MJ Research) Opticon, Opticon 2, and Chromo™ 4; Roche® LightCycler® 480 System; and Eppendorf Mastercycler® ep *realplex* Instruments without ROX filter set.

| Catalog no. | Size |
|---|---|
| 330500 | For 200 25-µl reactions (2.5 ml) |
| 330509 | For 2000 25-µl reactions (25 ml) |

### RT² qPCR Primer Assays

# Complementary Products

## XpressRef Universal Total RNA

Universal RNA to control PCR conditions is available from the following species:

- Human XpressRef Universal Total RNA (cat. no. 338112)
- Mouse XpressRef Universal Total RNA (cat. no. 338114)
- Rat XpressRef Universal Total RNA (cat. no. 338116)

# Protocol

Please read through this entire protocol before beginning your experiment. RNA samples are very sensitive to RNase digestion; therefore, wear gloves and maintain an RNase-free work area while performing this protocol.

## Considerations of RNA amount to be used

The RT² HT First Strand Kit yields results with as little as 25 ng or as much as 5 µg total RNA per well reaction. 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.

**Important**: Use a consistent amount of total RNA for all samples in a single experiment to be characterized and compared.

1. Remove the tubes of Buffer GE2 and BC4 Reverse Transcriptase Mix from -20°C storage and thaw on ice.
2. Briefly centrifuge the tubes after thawing. Keep the tubes on ice until they are used.
3. Transfer 750 µl of the Buffer GE2 to one of the 5-ml reagent reservoirs included. For best results, use a pipette to transfer the Buffer GE2 into the reagent reservoir. Decanting the Buffer GE2 from the tube into the reservoir is not recommended.
4. Add 6 µl of Buffer GE2 from the reagent reservoir to each well of the empty 96-well plate with a pipette.
5. Add 8 µl of a RNA sample to each well with a pipette and mix by pipetting up and down. Completely seal the plate with the foil adhesive sealing film.

   **NOTE**: The amount of total RNA per well should be consistent.

6. Centrifuge the plate at 1000 × g for 1 minute.
7. Incubate plate with compression mat on top at 37°C for 5 minutes in a thermal cycler (or room temperature for 10 minutes).
8. Carefully remove the foil seal.
9. Transfer 750 µl of the BC4 Solution (RT Master Mix) to the other included 5-ml reagent reservoir. For best results, use a pipette to transfer the BC4 Solution into the reagent reservoir. Decanting the BC4 Solution from the tube into the reservoir is not recommended.
10. Transfer 6 µl of BC4 Reverse Transcriptase Mix from the reagent reservoir to each well of the 96-well plate, and mix by pipetting up and down. Completely seal the plate with a new piece of foil adhesive sealing film.
11. Centrifuge the plate at 1000 × g for 1 minute.
12. Perform the reverse transcription (this step may be performed in any thermal cycler).
    - 12a. Set up a program for 42°C (15 minutes), 95°C (5 minutes), and 4°C hold (forever).
    - 12b. Put the plate in the thermal cycler with the reusable compression mat on top of the plate.
    - 12c. Close lid of the thermal cycler and run the program.
13. Keep plate with finished reaction on ice until ready to use for real-time PCR, or at -20°C for long-term storage.
14. If performing analysis using RT² Profiler PCR Arrays or Primer Assays,
    - 14a. Thaw plate, and then centrifuge the plate at 1000 × g for 1 minute.
    - 14b. Transfer the cDNA to new tubes and add 91 µl $H_2O$.
    - 14c. Continue with protocol in the *RT² Profiler PCR Array Handbook* or *RT² qPCR Primer Assay 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/FAQ/FAQList.aspx.** 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 |
|---|---|
| **Real-time PCR Ct values are too high (>35 or not detectable)** | |
| a) Poor RNA quality | Be sure to perform all recommended quality control checks on the RNA sample. Poor quality RNA can inhibit enzyme activity during reverse transcription generating an insufficient amount of template during the first-strand cDNA synthesis reaction. |
| b) Not enough templates | Use more input RNA for reverse transcription especially if the lower end of the recommended range had been used previously.<br>Use a larger volume of template per reaction, but do not use more than 1.25 µl of template per 25-µl reaction. Remember to use the same volume of template in each reaction. |
| c) Incomplete/inefficient mixing of the reverse transcription reaction | Be sure to mix the reverse transcription reaction well by pipetting when BC4 Reverse Transcriptase Mix is added to samples that have been treated with the genomic DNA elimination procedure. |
| d) Incorrect temperature of reverse transcription reaction | Reverse transcription should be carried out at 42°C. Higher temperature will reduce activity of reverse transcriptase and affect cDNA yield. |
| e) Incomplete sealing during reverse transcription reaction | Be sure to completely seal the plate using the provided aluminum film to avoid reaction volume loss during RT. |
| **Genomic DNA contamination** | |
| a) Too much genomic DNA in your RNA samples | The genomic DNA elimination procedure provided by the RT² HT First Strand Kit can clean up to 200 ng genomic DNA for each RNA sample. RNA samples heavily contaminated with genomic DNA need to be re-purified by recommended RNA isolation methods (see page 5). |

| | | Comments and suggestions |
|---|---|---|
| b) | Incomplete/inefficient mixing of the genomic DNA elimination reaction | Be sure to mix genomic DNA elimination reaction well by pipetting when RNA samples are added to each well of Buffer GE2 Plate. |
| c) | Incomplete sealing during genomic DNA elimination reaction | Be sure to completely seal the plate using the provided aluminum film to avoid reaction volume loss. |

**Appearance of multiple PCR products (dissociation peaks or gel bands)**

| | | |
|---|---|---|
| a) | Primer dimers | Verify presence of primer dimers (<50 bp in size) by agarose gel electrophoresis. Be sure to use the appropriate RT² SYBR Green qPCR Mastermix to prevent the appearance of primer dimers. |

# Appendix A: RNA Quality Control

High quality RNA is ESSENTIAL for obtaining good real-time PCR results.

The most important prerequisite for any gene expression analysis experiment is consistent, high-quality RNA from every experimental sample. Therefore, the sample handling and RNA isolation procedures are critical to the success of the experiment. Residual traces of proteins, salts or other contaminants will either degrade the RNA or decrease the efficiency of (if not block completely) the enzyme activities necessary for optimal reverse transcription and real-time PCR performance.

For best results from the RT-PCR, all RNA samples should also demonstrate consistent quality according to the following criteria:

**RNA Concentration and Purity by UV Spectrophotometry**

**NOTE**: Prepare dilutions and measure absorbance in 10 mM Tris, pH 8.0 buffer. The spectral properties of nucleic acids are highly dependent on pH.

- $A_{260}$:$A_{230}$ ratio should be greater than 1.7.
- $A_{260}$:$A_{280}$ ratio should be greater than 2.0.
- Concentration by $A_{260}$ should be greater than 4 µg/ml total RNA

**Ribosomal RNA band integrity**

Electrophorese a fraction of each RNA sample on a denaturing agarose gel or on an Agilent BioAnalyzer using an RNA 6000 Nano LabChip®, and verify that there is a sharp distinction at the small side of both the 18S and 28S ribosomal RNA (rRNA) bands or peaks. Any smearing or shoulder to the rRNA bands or peaks indicates that degradation has occurred in the RNA sample.

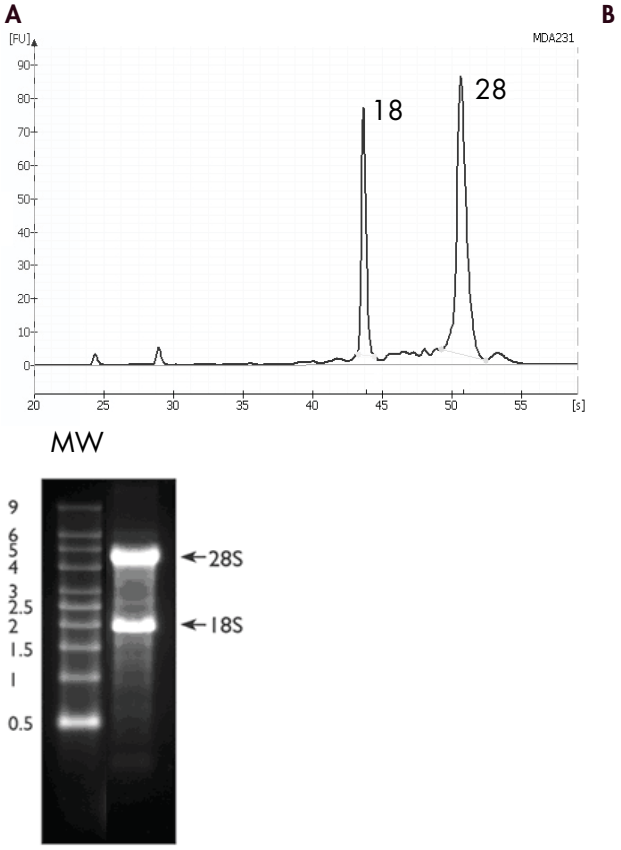


**Figure 1. Good Ribosomal RNA Band Integrity Is Important for Optimal PCR Array Results.**
Panel **A** displays an Agilent BioAnalyzer electropherogram of a high-quality total RNA preparation showing sharp peaks without shoulders (especially to the left of each peak) for the 18S and 28S ribosomal RNA (left to right). Panel **B**, right-hand lane, displays an analysis of the same high-quality total RNA preparation by agarose gel electrophoresis demonstrating sharp bands (especially at the bottom of each band) for the 28S and 18S ribosomal RNA (top to bottom).

Because some contaminants are difficult to detect by simply looking at RNA integrity and can be missed by UV spectrophotometry, it is essential to choose the proper RNA isolation method for your biological sample as described above.

# Appendix B: Real-Time RT$^2$ qPCR Primer Assay

**NOTE**: Accurate pipetting is very critical for the success of this protocol. Be sure that all of your micro-pipettors are calibrated before beginning this procedure. Also, be sure not to introduce any bubbles into the wells of the PCR reaction.

**1. Setup**

1a. Experimental Samples

To insure the consistency of your results and that each experimental sample yields a reliably detectable $C_T$ value, we recommend using undiluted template and a 1:5 dilution of your template in separate reactions. Also, prepare either duplicate or triplicate reactions for each template at each concentration.

For every experimental sample, prepare 1 set of reactions for every gene of interest and for a single housekeeping gene or a set of housekeeping genes to normalize your raw data. Choose housekeeping gene(s) known not to change their expression under your experimental conditions.

1b. Positive and Negative Controls

Prepare a positive control reaction using template known to represent the genes of interest such as template generated from XpressRef Universal Total RNA.

To control for DNA contamination introduced during reaction setup, prepare a negative control reaction replacing template with water, the so-called no template control (NTC).

To control for genomic DNA contamination, perform 1 assay for each gene of interest and each housekeeping gene using an equivalent volume of product from the No Reverse Transcription (NRT) reaction performed for each RNA sample.

1c. Optional Standard Curve Method

Generate 1 standard curve for each gene of interest. If you plan to normalize your data to a housekeeping gene or a set of housekeeping genes, also generate a standard curve for the housekeeping gene(s).

To generate a standard curve, prepare a five-point series of five- or ten-fold dilutions in duplicate using a template known to represent the genes of interest, such as template generated from XpressRef Universal Total RNA.

2. **Polymerase Chain Reactions**

**NOTE**: The use of RT² SYBR Green qPCR Mastermixes is absolutely critical for obtaining accurate results with the RT² qPCR Primer Assays. Be sure to use the correct master mix for your instrument before continuing this protocol (see page 8.)

For each 25-µl PCR, mix the following components in each well of a PCR 96-well plate:

| Component | Volume |
|---|---|
| RT² qPCR Mastermix | 12.5 µl |
| dd$H_2O$ | 10.5 µl |
| of either diluted or undiluted template | 1.0 µl |
| RT² qPCR Primer Assay | 1.0 µl |
| **Final volume** | **25.0 µl** |

Use **Program #1**, a two-step cycling program, for all of the following instrumentation:

- All ABI Instruments (7000, 7300, 7500, 7700 and 7900HT)
- BioRad iCycler, MyiQ cycler, and iQ5 real-time PCR detection systems
- All Stratagene Instruments (Mx3000P®, Mx3005P®, and Mx4000P®)

- Eppendorf Mastercycler ep *realplex* and Roche LightCycler 480

| Cycles | Duration | Temperature |
|---|---|---|
| 1 | 10 min* | 95°C |
| 40 | 15 sec | 95°C |
| | 1 min† | 60°C |

* The 10-minute step at 95°C is required to activate the HotStart DNA polymerase.

Use **Program #2**, a three-step cycling program, for all other instruments:
For example, the BioRad (MJ Research) Opticon, Opticon 2, and Chromo 4.

| Cycles | Duration | Temperature |
|---|---|---|
| 1 | 10 min* | 95°C |
| | 15 sec | 95°C |
| 40 | 30 to 40 sec†, ‡ | 55°C |
| | 30 sec | 72°C |

* The 10-minute step at 95°C is required to activate the HotStart DNA polymerase.

† Detect and record SYBR Green fluorescence from every well during the annealing step of each cycle.

‡ Different instruments need different lengths of time to detect the fluorescent signal. Choose the annealing step (55°C) time appropriate for your instrument.

# Ordering Information

| Product | Contents | Cat. no. |
|---|---|---|
| RT² HT First Strand Kit (96) | Reagents and plasticware for 96 reverse transcription reaction | 330411 |

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.

| Document Revision History | |
|---|---|
| R2<br>11/2018 | Updated template.<br>Updated data analysis procedure. |