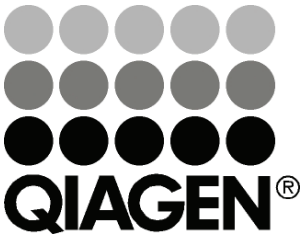
March 2011

RT^2 Easy First Strand Handbook

For cDNA synthesis



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- Purification of DNA, RNA, and proteins
- Nucleic acid and protein assays
- microRNA research and RNAi
- Automation of sample and assay technologies

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Product Use Limitations

The RT^2 Easy 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.

CONTENTS

I. Background and Introduction 4

II. Materials Provided 5

III. Principle 6

IV. Additional Equipment and Reagents 7

V. Complementary Products 8

VI. Protocol 9

VII. Troubleshooting and Frequently Asked Questions 10

Appendix A: RNA Quality Control 11

Appendix B: Real-Time RT^2 qPCR Primer Assay 12

I. Background and 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 Easy 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 multiple RNA samples for reverse transcription. 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, and other commercially available primer assays.

Benefits of the RT² Easy First Strand Kit:

- **Easy and Fast:** Everything included in one kit to eliminate genomic DNA and complete first strand cDNA synthesis for your RNA samples
- **Eliminate False Positives:** Optimized DNA removal buffer prevents false positive signals due to amplification of genomic DNA contamination

II. Materials Provided:

Kits Contents

RT^2 Easy First Strand Kit	
Catalog no.	330421
Number of 20 µl reverse-transcription reactions	12
Components	**Volume Included**
Buffer GE2 (gDNA elimination buffer)	80 µl
BC5 Reverse Transcriptase Mix	80 µl

Storage Conditions

Buffer GE2 and BC5 Reverse Transcriptase Mix 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.

Shelf Life

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

III. Principle:

The RT^2 Easy First Strand Kit procedure comprises 2 steps: elimination of genomic DNA contamination, and reverse transcription, which enable fast and easy handling of multiple RNA samples.

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 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.

IV. Additional Equipment and Reagents:

A. For RNA Isolation:

RNeasy® Mini Kit (cat. no. 74104) for individual samples

B. For First Strand cDNA Synthesis

Regular thermal cycler or water bath

C. For Real-time PCR

a. 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
Eppendorf® Mastercycler® ep *realplex* Instruments with ROX filter set

Catalog Number	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 Number	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 Chromo4™
Roche® LightCycler® 480 System
Eppendorf Mastercycler ep *realplex* Instruments without ROX filter set

Catalog Number	Size
330500	For 200 25-µl reactions (2.5 ml)
330509	For 2000 25-µl reactions (25 ml)

b. RT² qPCR Primer Assays or other commercially available primer assays

V. 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)

VI. 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 RT^2 Easy 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. It is also important to use a consistent amount of total RNA for all samples in a single experiment to be characterized and compared.

1. Prepare a separate Genomic DNA Elimination mixture for each RNA sample:

Total RNA:	25.0 ng to 5.0 µg
Buffer GE2 (gDNA elimination buffer):	6.0 µl
RNase-free H_2O to a final volume of:	14.0 µl

2. Incubate at 37°C for 5 min, and immediately place on ice for at least 1 minute.

3. Add 6 µl of the BC5 Reverse Transcriptase Mix to each 14-µl Genomic DNA Elimination Mixture for a final volume of 20 µl.

4. Incubate at 42°C for exactly 15 min and then immediately stop the reaction by heating at 95°C for 5 minutes.

5. Hold the finished reaction on ice until ready to use for real-time PCR, or place at -20°C for long-term storage.

Note: For real-time PCR, the cDNA should not be more than 5% of the total PCR volume.

VII. TROUBLESHOOTING GUIDE AND FAQ:

A. Real-time PCR C_t values are too high (> 35 or not detectable)

1. 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.

2. Not enough template:

   a. Use more input RNA for reverse transcription especially if the lower end of the recommended range had been used previously.
   b. 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.

3. Incomplete/inefficient mixing of the reverse transcription reaction:

   Be sure to mix reverse transcription reaction well by pipetting when BC5 Reverse Transcriptase Mix is added to samples that have been treated by the genomic DNA elimination procedure.

4. Incorrect temperature of reverse transcription reaction:

   Reverse transcription should be carried out at 42°C. Higher temperature will reduce the activity of reverse transcriptase and affect cDNA yield.

B. Genomic DNA contamination

1. Too much genomic DNA in your RNA samples

   The genomic DNA elimination procedure provided by RT^2 Easy 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 7).

2. Incomplete/inefficient mixing of the genomic DNA elimination reaction:

   Be sure to mix genomic DNA elimination reaction well by pipetting when Buffer GE2 (gDNA elimination buffer) is added to RNA samples.

C. Appearance of multiple PCR products (dissociation peaks or gel bands):

1. Primer Dimers:

   Verify presence of primer dimers (< 50 bp in size) by agarose gel electrophoresis. Be sure to use the appropriate RT^2 SYBR Green qPCR Mastermix to prevent the appearance of primer dimers.

Appendix:

A. RNA Quality Control:

High quality RNA is <u>ESSENTIAL</u> 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:

a. 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.*

i) A_{260}:A_{230} ratio should be greater than 1.7.
ii) A_{260}:A_{280} ratio should be greater than 2.0.
iii) Concentration by A_{260} should be greater than 4 μg / ml total RNA

b. 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.

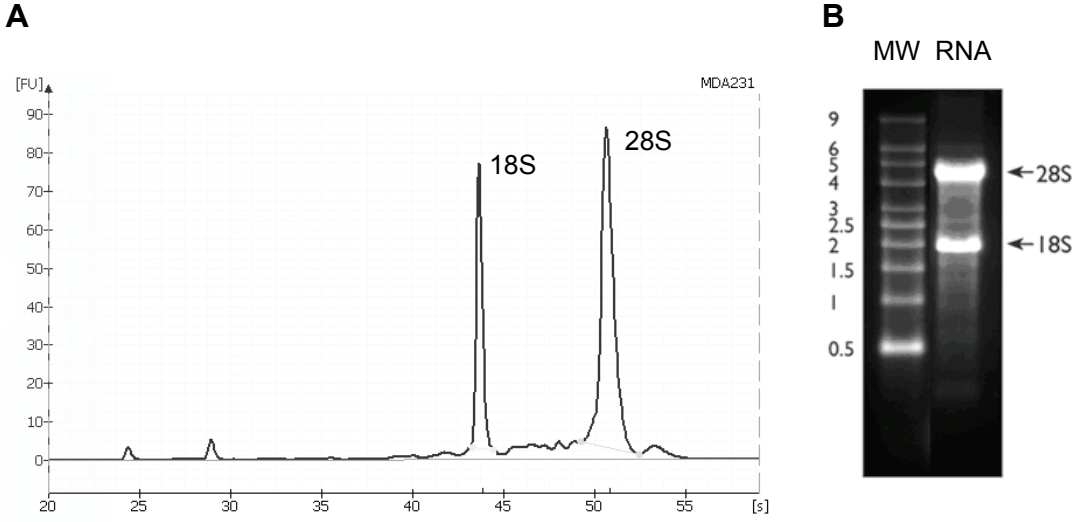

Figure 1: Good Ribosomal RNA Band Integrity Is Important for Optimal PCR Array Results.
Panel **A** displays an Agilent Bioanalyzer electropherogram of a high-quality total RNA preparation showing sharp peaks without shoulders (especially to the left of each peak) for the 18S and 28S ribosomal RNA (left 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.

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 to not introduce any bubbles into the wells of the PCR reaction.*

1. **Setup:**

   a. 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 one 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 to not change their expression under your experimental conditions.

   b. 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 one 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.

   c. Optional Standard Curve Method:

Generate one 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 RT^2 SYBR Green qPCR Mastermixes is absolutely critical for obtaining accurate results with the RT^2 qPCR Primer Assays. Be sure to use the correct master mix for your instrument before continuing this protocol (See Page 7.)*

For each 25-µl PCR, mix the following components in a nuclease-free PCR tube:

12.5 µl RT^2 qPCR Mastermix
10.5 µl ddH_2O
1.0 µl of either diluted or undiluted template
1.0 µl RT^2 qPCR Primer Assay
25.0 µl final volume

Place the tube in your real-time thermal cycler.
Enter and run the appropriate program for your real-time instrument:

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 minutes[1]	95 °C
40	15 seconds	95 °C
	1 minute[2]	60 °C

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 minutes[1]	95°C
40	15 seconds	95°C
	30 to 40 seconds[2,3]	55°C
	30 seconds	72°C

[1] The 10-minute step at 95°C is required to activate the HotStart DNA polymerase.
[2] Detect and record SYBR Green fluorescence from every well during the annealing step of each cycle.
[3] 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.
RT^2 Easy First Strand Kit (12)	Reagents for 12 reverse transcription reactions	330421

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