PROTOCOL

ambion® | RNA
by life technologies™

Fast SYBR® Green Cells-to-CT™ Kit

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life technologies™

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Part Number 4403786 Rev. C
03/2011

Fast SYBR® Green Cells-to-CT™ Kit

Protocol

I. Introduction

IMPORTANT

Before using this product, read and understand the "Safety Information" in the appendix in this document.

A. Product Description and Background

The Fast SYBR® Green Cells-to-CT™ Kit includes reagents and enzyme mixtures for reverse transcription (RT) and real-time PCR directly from cultured cell lysates, without isolating RNA.

RT-PCR with real-time detection of amplification products is a robust, simple, and quantitative way to measure mRNA levels in biological samples. Traditionally, the first step in gene expression experiments has been to recover pure RNA from experimental samples. Even using the quickest and simplest techniques, however, RNA isolation is fairly time consuming, often requiring 30 minutes or more of hands-on sample manipulation. Furthermore, with small samples, it is subject to loss of RNA. Cells-to-CT™ technology enables reverse transcription of lysates from 10–10^5 cultured cells without isolating or purifying RNA. Real-time PCR analysis is carried out directly afterwards. Eliminating the RNA isolation step substantially expedites and simplifies gene expression analysis of cultured cells.

Cells-to-CT lysates exhibit sensitivity and specificity similar to that from purified RNA in real-time RT-PCR. The lysis procedure simultaneously prepares cell lysates for RT-PCR and removes genomic DNA, in under ten minutes. The lysis step is simple to automate with robotic platforms for high-throughput processing of 96- or 384-well plates, because it takes place entirely at room temperature. The procedure is also economical; there are only a few pipetting steps and, with cells grown in 96- or 384-well plates, no sample transfers.

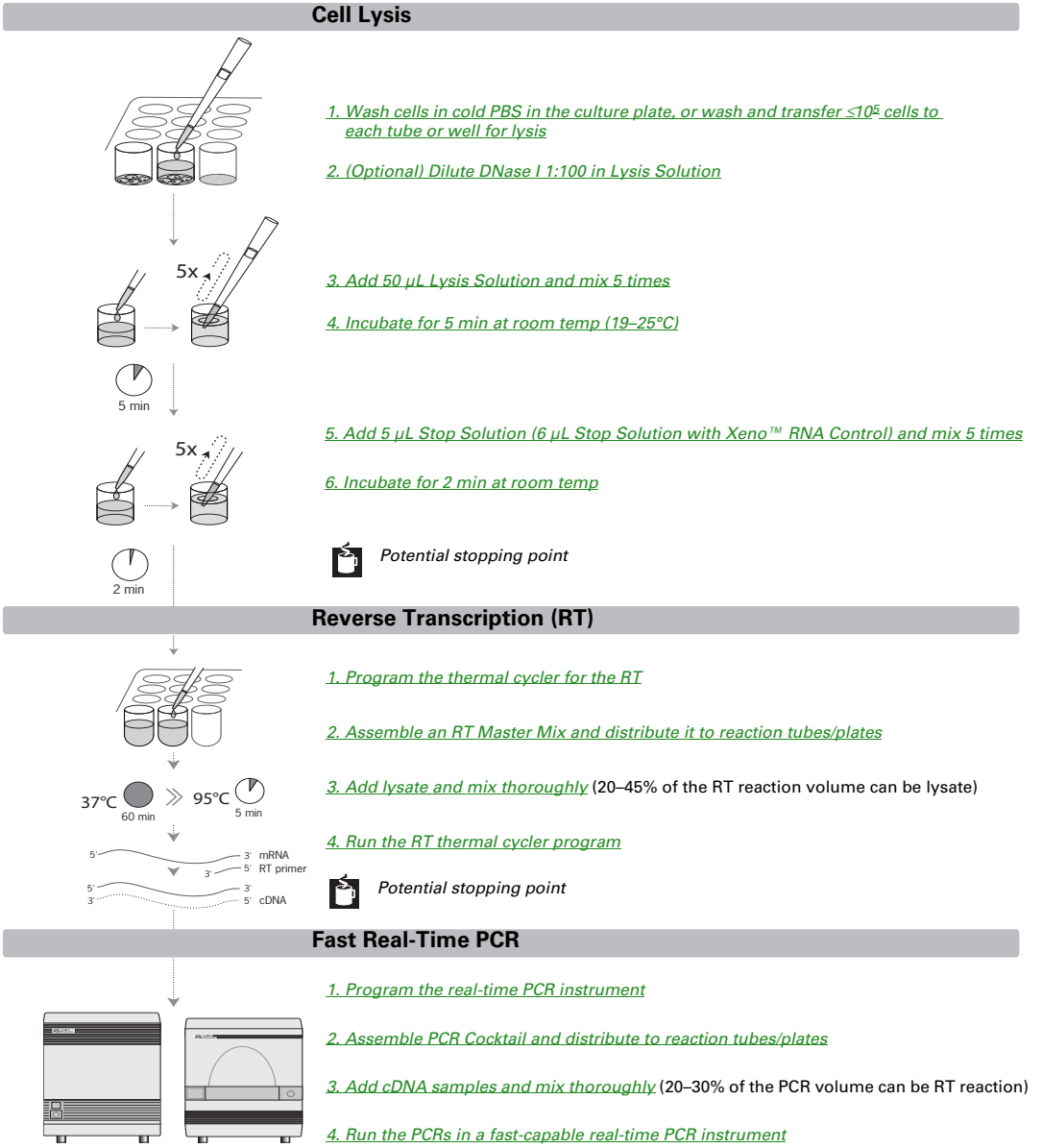
Procedure overview

The Fast SYBR Green Cells-to-CT Kit procedure is shown in Figure 1 and described below.

- First, 10–10^5 cultured cells are washed with phosphate-buffered saline (PBS), mixed with Lysis Solution, and incubated at room temperature for 5 min. Cells are lysed during this incubation and RNA is released into the Lysis Solution, which contains reagents to inactivate endogenous RNases. If DNase I is added to the Lysis Solution (optional), genomic DNA is also degraded at this step.
- Next, Stop Solution is mixed into the lysate to inactivate the lysis reagents so that they will not inhibit the RT or PCR.
- Cell lysates are reverse transcribed to synthesize cDNA using a convenient 20X RT Enzyme Mix and 2X SYBR RT Buffer.

- Finally, the cDNA is amplified by real-time PCR using the included Fast SYBR Green PCR Master Mix and the PCR primer set for the target of interest (user supplied).

Figure 1. Fast SYBR® Green Cells-to-CT™ Procedure Overview



SYBR® Green Cells-to-CT™ Control Kit

The SYBR® Green Cells-to-CT Control Kit (P/N 4402959, available separately) is designed for use with the *Power* SYBR Green Cells-to-CT Kits and Fast SYBR Green Cells-to-CT Kits. It includes Xeno™ RNA Control, an RNA transcript that has no homology to any known sequences, and a PCR primer pair to detect the Xeno RNA Control target. It also includes a set of PCR primers for the highly expressed endogenous control gene, β-actin. These PCRs are designed to be run in parallel with amplification for the gene(s) of interest (not in multiplex), to serve as positive controls for RT and real-time PCR. Figure 2 shows an experiment using HeLa cells and the β-actin PCR primers to evaluate the dynamic range of the Fast SYBR® Green Cells-to-CT™ Kit.

Applications

The Fast SYBR® Green Cells-to-CT™ Kit can be used in any SYBR Green-based real-time RT-PCR application to analyze mRNA from cultured cells. The kit is well suited for large experiments, such as real-time RT-PCR analysis of gene expression in differentially treated cell cultures or RNAi screening experiments using siRNA to modulate gene expression. Other applications include screening a library of compounds for their effects on mRNA expression, following the regulation of mRNA as cells are treated with increasing concentrations of a particular chemical, or evaluating the expression of mRNA in time course experiments.

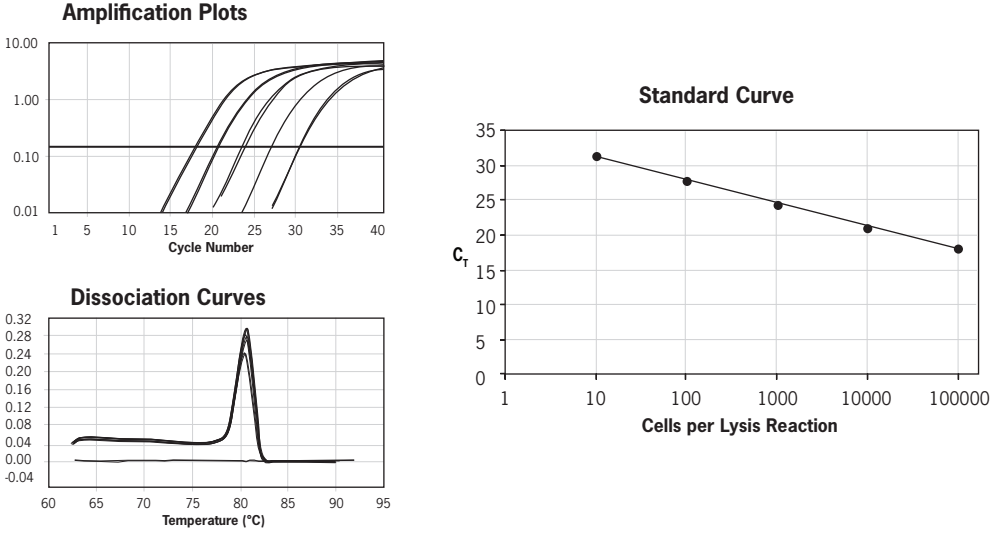


Figure 2. Real-time RT-PCR Using the Fast SYBR® Green Cells-to-CT™ Kit

A dilution series of 10–100,000 HeLa cells was processed in duplicate with the Fast SYBR® Green Cells-to-CT™ Kit. The endogenous control gene β-actin was amplified from the cDNA in 20 µL reactions. The amplification plots are shown on the top left and the dissociation curve is shown on the bottom left. The dissociation curve indicates that the reactions are free of primer-dimer or any other spurious products. The standard curve (on the right) shows the threshold cycle (C_T) compared to the input number of cells. Amplification was linear over a cell input range of 10–10^5 cells per lysis.

Cell type compatibility

Table 1 lists the cell types that have been shown to be compatible with the Cells-to-CT technology.

Table 1. Cell Types Compatible with Cells-to-CT™ Technology

Cell Line	Growth	Source Species	Source Tissue
A549	adherent	*H. sapiens*	Lung Carcinoma
BJ	adherent	*H. sapiens*	Foreskin fibroblast
CHO-K1	adherent	*C. griseus* (hamster)	Ovary
COS-7	adherent	*C. aethiops* (monkey)	Kidney
DU-145	adherent	*H. sapiens*	Prostate Carcinoma
HEK-293	adherent	*H. sapiens*	Kidney
HeLa	adherent	*H. sapiens*	Cervical Adenocarcinoma
HepG2	adherent	*H. sapiens*	Liver Carcinoma
Huh-7	adherent	*H. sapiens*	Liver carcinoma
Jurkat	suspension	*H. sapiens*	Acute T-Cell Leukemia
K-562	suspension	*H. sapiens*	Chronic Myelogenous Leukemia
ME-180	adherent	*H. sapiens*	Cervical Epidermoid Carcinoma
NCI-H460	adherent	*H. sapiens*	Large Cell Lung Cancer
Neuro 2A	adherent	*M. musculus* (mouse)	Brain blastoma
NIH/3T3	adherent	*M. musculus* (mouse)	Embryonic Fibroblast
PC-12	adherent	*R. norvegicus* (rat)	Adrenal Pheochromocytoma
Primary Hepatocytes	adherent	*H. sapiens*	Liver
PT-K75	adherent	*S. scrofa* (pig)	Nasal Turbinate Mucosa
Raji	suspension	*H. sapiens*	B Lymphocyte
SK-N-AS	adherent	*H. sapiens*	Brain Neuroblast
SK-N-SH	adherent	*H. sapiens*	Brain Fibroblast
U-87 MG	adherent	*H. sapiens*	Brain Glioblastoma
U-2 OS	adherent	*H. sapiens*	Bone osteosarcoma

B. Materials Provided with the Kit and Storage Conditions

The Fast SYBR® Green Cells-to-CT™ Kit is available in sizes for lysis of 100 or 400 samples.

- ***100 reaction*** size (P/N 4402956): For each lysis reaction, RT reagents for one reverse transcription reaction (RT) plus one minus-RT control are included, and Fast SYBR Green PCR Master Mix for 500, 20 µL PCRs.
- ***400 reaction*** size (P/N 4402957): For each lysis reaction, RT reagents for one reverse transcription reaction (RT) and one minus-RT control are included, and Fast SYBR Green PCR Master Mix for 2000, 20 µL PCRs.

P/N4402956 100 rxn	P/N 4402957 400 rxn	Component	Storage
500 µL	2 x 1 mL	Stop Solution	–20°C
55 µL	220 µL	DNase I	–20°C
275 µL	1.1 mL	20X RT Enzyme Mix	–20°C
5.5 mL	22 mL	Lysis Solution	4°C
5.5 mL	22 mL	2X SYBR® RT Buffer	4°C
5 mL	4 x 5 mL	Fast SYBR® Green PCR Master Mix	4°C
2 x 5 mL	50 mL	Nuclease-free Water	any temp*

* Store Nuclease-free Water at –20°C, 4°C, or room temperature.

Note that PCR primers needed for real-time PCR for your target(s) of interest are not included with the Fast SYBR Green Cells-to-CT Kit.

NOTE

The product is shipped on dry ice, but upon receipt the kit components should be stored at the temperatures indicated in the table above. Storage at –20°C should be in a non-frost-free freezer.

C. Materials Not Provided with the Kit

Lab equipment and supplies

- General laboratory equipment including vortex mixer, microcentrifuge, and pipettors.
- Nuclease-free pipette tips, nuclease-free microcentrifuge tubes and/or U-bottom 96-well plates (for cells ***not*** grown in 96- or 384-well culture plates).
- Fast real-time PCR tubes or multiwell plates appropriate for your instrument e.g., Applied Biosystems MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL, P/N 4346906.
- Thermal cycling instrument. The Cells-to-CT Kits were developed using Applied Biosytems thermal cyclers for the RT reaction and real-time PCR instruments for the PCR. However, the technology is expected to be compatible with any thermal cycler for the RT reaction and with any real-time PCR system compatible with SYBR Green fluorescent dye and capable of fast cycling conditions. Fast SYBR Green Cells-to-CT reactions can be run on any currently available Applied Biosystems instrument platforms, including the following:

  Reverse transcription
  - GeneAmp® PCR System 9700
  - Veriti™ 96-Well Thermal Cycler

Real-Time PCR

- Applied Biosystems 7900 HT Fast Real-Time PCR System with Fast 96-Well Block Module or 384-Well Block Module. (An upgraded power supply is needed to use the 384-well block in fast mode. Download the User Bulletin "Performing Fast Gene Quantitation with 384-Well Plates, P/N 4369584" from www.appliedbiosystems.com for instructions on fast cycling with the 384-well block.)
- Applied Biosystems 7500 Fast Real-Time PCR System.
- StepOne™ or StepOnePlus™ Real-Time PCR Systems.

Reagents

- Phosphate-buffered saline (PBS), for example, diluted from Ambion 10X PBS (P/N AM9624, AM9625).
- PCR primers for real-time PCR of the target(s) of interest. See "PCR Primer Design Suggestions" on page 17.
- (optional) SYBR® Green Cells-to-CT™ Control Kit (P/N 4402959).

Software

(optional) Applied Biosystems Primer Express Software (P/N 4330710), for primer design for your target sequence of interest.

D. Related Products

SYBR® Green Cells-to-CT™ Control Kit P/N 4402959	Designed for use with *Power* SYBR® Green and Fast SYBR Green Cells-to-CT™ kits, the SYBR Green Cells-to-CT Control Kit includes Xeno™ RNA Control, an RNA transcript that has no homology to any known sequence, and a corresponding set of PCR primers. It also includes a PCR primer set for the highly expressed endogenous control gene β-Actin. These reactions are designed to be run in parallel with PCR(s) for the gene(s) of interest, to serve as positive controls for reverse transcription and real-time PCR.
Applied Biosystems Real-Time PCR Instruments See product catalog for P/Ns	The Applied Biosystems real-time platforms, which include the Applied Biosystems 7500 Fast, 7900HT Fast, StepOne™, and StepOnePlus™ Real-Time PCR Systems, provide cutting-edge tools while making real-time PCR more accessible than ever. These systems are easy to use with next generation software and the flexibility to run the real-time chemistry of your choice.
Applied Biosystems Primer Express Software P/N 4363991	Primer Express® Software v3.0 allows you to design your own primers and probes using TaqMan® and SYBR® Green I dye chemistries for absolute/relative quantitation and allelic discrimination (SNP) real-time PCR applications. Developed specifically for use with the Applied Biosystems 7500 Fast and 7900HT Fast Real-Time PCR Systems, and the 7300 and 7500 Real-Time PCR Systems.
RNase*Zap*® Solution P/N AM9780, AM9782, AM9784	RNase*Zap*® RNase Decontamination Solution is simply sprayed, poured, or wiped onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water eliminates all traces of RNase and RNase*Zap* Solution.
RNase-free Tubes & Tips See product catalog for P/Ns	Ambion® RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free. For more information, see our product catalog at www.invitrogen.com/ambion.

RT-PCR Grade Water P/N AM9935	Ambion® RT-PCR Grade Water is certified free of nucleases, and free of nucleic acid contamination that may cause false-positive signals in RT-PCR. The RT-PCR Grade Water is ready to use and requires no preparation, mixing, or autoclaving. Like all Ambion water products, RT-PCR Grade Water is deionized, autoclaved, and sterile filtered. Each lot is subjected to 2 rounds of rigorous quality control testing before being certified nuclease-free.
Plastic Consumables for PCR See product catalog for P/Ns	Applied Biosystems MicroAmp® disposables are optimized to provide unmatched temperature accuracy and uniformity for fast, efficient PCR amplification. The plastics are compatible with various Applied Biosystems platforms, including real-time PCR instruments. Choose your format based on throughput requirements or your thermal cycler block.
DNA*Zap*™ Solution P/N AM9890	DNA*Zap*™ PCR DNA degradation solution consists of two solutions that are innocuous separately but, when mixed, are able to degrade instantly high levels of contaminating DNA and RNA from surfaces.

II. Procedure

A. Suggested Controls and Replicates

Biological and technical replicates

In this protocol, we define *biological replicates* as separate lysis reactions and *technical replicates* as separate PCRs. The minimum and recommended number of replicates are the following:

- Biological replicates: 2–4
- Technical (PCR) replicates: 2–4

Negative controls for RT-PCR

Minus-RT control

Minus-RT controls contain all the RT reaction components except the 20X RT Enzyme Mix (substitute water); minus-RT controls demonstrate that the template for the PCR was cDNA, and not genomic DNA.

No-template control

No-template controls (NTC) contain all the PCR components except the cell lysate (substitute water). If the no-template control yields a fluorescent signal, the RT or PCR reagents may be contaminated with DNA, for example, PCR product from previous reactions.

No-amplification control

Because fluorescent contaminants can cause false-positive results in real-time PCR using SYBR® Green Dye, it may be necessary to include a No-amplification Control (NAC) that contains sample and all the PCR components except the Fast SYBR Green PCR Master Mix. If the absolute fluorescence of the NAC is greater than that of the NTC after PCR, fluorescent contaminants may be present in the sample or in the heat block of the thermal cycler.

B. Cell Lysis

NOTE

These instructions describe using fresh cultured cells. If your experiment requires frozen cultured cells, we recommend washing the cells in cold PBS as described in step 1 (below) before freezing. When you are ready to start the procedure, allow the cells to thaw on ice, and begin the procedure at step 2.

Before you start

- Thaw Stop Solution, invert or flick the tube several times to mix thoroughly (do ***not*** vortex), and place the solution on ice.
- Chill 1X PBS to 4°C.

1. Wash cells in cold PBS in the culture plate, or wash and transfer ≤10^5 cells to each tube or well for lysis

IMPORTANT

The maximum number of cells that can be used in this procedure varies somewhat according to cell type, but is generally 10^5 cells. Instructions for a pilot experiment to determine the maximum number of cells per reaction is outlined in section IV.B starting on page 18. Using too many cells per lysis reaction may result in incomplete lysis and/or inhibition of RT-PCR.

Adherent cells grown in 96- or 384-well plates

It is important to start with cells that have been cultured until they are fully adhered to the plate; otherwise, they will be lost during washing.

a. Estimate (or count) the number of cells per well. Make sure that each well contains 10–10^5 cells.

b. Aspirate and discard the culture medium from the wells.

c. Add 50 µL of cold (4°C) 1X PBS to each well.

d. Aspirate the PBS from the well. Remove as much PBS as possible without disturbing the cells. ***Proceed to step 2.***

Cells grown in other vessels (adherent and suspension cells)

a. Adherent cells only (for suspension cells, start at step b): Detach cells using the subculturing method routinely employed in your laboratory for the cell type. If trypsin is used, inactivate it before proceeding.

b. Count then gently pellet the cells, aspirate and discard the growth medium, and place cells on ice.

NOTE

Alternatively, cells can be split into aliquots for individual lysis reactions before washing in PBS.

c. Wash cells in cold (4°C) PBS by resuspending them in ~0.5 mL PBS per 10^6 cells (or ~50 µL PBS for ≤10^5 cells). Gently pellet the cells, then aspirate and discard as much of the PBS as possible without disturbing the cell pellet. Place the cells on ice.

d. Resuspend cells in fresh, cold 1X PBS so that 5 µL will contain the desired number of cells for a single lysis reaction (10–10^5 cells/lysis).

e. Split the cell suspension into individual lysis reactions: distribute 5 µL of the suspension to wells of a U-bottom multiwell plate or microcentrifuge tubes. Place the cells on ice.

2. (Optional) Dilute DNase I 1:100 in Lysis Solution

To remove genomic DNA during cell lysis, determine the volume of Lysis Solution needed for the experiment, and dilute DNase I into the Lysis Solution at 1:100 for use in the next step. Include ~10% overage; examples are shown in Table 2 below:

Table 2. Preparation of Lysis Solution Containing DNase I

Component	each rxn	96 rxns	384 rxns
Lysis Solution	49.5 µL	5.23 mL	20.91 mL
DNase I	0.5 µL	52.8 µL	211 µL

3. Add 50 µL Lysis Solution and mix 5 times

a. Add 50 µL Lysis Solution (with or without DNase I) to each sample.

b. Mix the lysis reaction by pipetting up and down 5 times.
To avoid bubble formation, mix with the pipettor set at 35 µL and expel the solution without emptying the pipette tip completely.

NOTE

Lysis Solution and Cells-to-CT lysates may appear cloudy at room temperature—this is expected.

4. Incubate for 5 min at room temp (19–25°C)

Incubate the lysis reactions for 5 min at room temperature (19–25°C).

5. Add 5 µL Stop Solution (6 µL Stop Solution with Xeno™ RNA Control) and mix 5 times

a. (Optional) To include an endogenous control using the SYBR Green Cells-to-CT Control Kit, prepare Stop Solution containing Xeno™ RNA Control for the samples in the experiment at hand. Add 1 µL Xeno RNA Control per 5 µL Stop Solution.

b. Pipet 5 µL of Stop Solution alone or 6 µL of Stop Solution with Xeno RNA Control directly into each lysis reaction.

Touch the surface of the lysate with the opening of the pipette tip to ensure that all of the Stop Solution is added to the lysate.

c. Mix the lysis reaction by pipetting up and down 5 times.
To avoid bubble formation, mix with the pipettor set at 35 µL and expel the solution without emptying the pipette tip completely.

NOTE

To dispense Stop Solution using a multichannel pipettor, pipet Stop Solution from a set of strip tubes or adjacent wells in a 96-well plate rather than from a reagent reservoir. If desired, store unused Stop Solution frozen in properly sealed strip tubes.

IMPORTANT

It is very important to thoroughly mix the Stop Solution into the lysate.

6. Incubate for 2 min at room temp

Incubate for 2 min at room temp (19–25°C).
Do not allow Cells-to-CT lysates to remain at room temp for longer than 20 min after adding the Stop Solution.

STOPPING POINT

Lysates can be stored on ice for ≤2 hr, or at –20°C or –80°C for ≤5 months.

C. Reverse Transcription (RT)

1. Program the thermal cycler for the RT

Program the thermal cycler as shown in Table 3.

Table 3. Thermal Cycler Settings for RT

	Stage	Reps	Temp	Time
Reverse transcription (hold)	1	1	37°C	60 min
RT inactivation (hold)	2	1	95°C	5 min
Hold	3	1	4°C	indefinite

2. Assemble an *RT Master Mix* and distribute it to reaction tubes/plates

a. Calculate the number of RT reactions in the experiment. Using Table 4 below, assemble an ***RT Master Mix*** for all the reactions plus ~10% overage in a nuclease-free microcentrifuge tube on ice.
Table 4 shows the reaction setup using the recommended amount of lysate in each RT reaction, 10 µL. Up to 45% of the RT reaction volume (22.5 µL) can be Cells-to-CT lysate; adjust the volume of Nuclease-free Water accordingly.


NOTE

If a 50 µL RT reaction will not provide enough material for all of the planned PCRs, the RT reaction can be scaled up proportionally.

Table 4. RT Master Mix: 50 µL Final Reaction Volume

Component	Each rxn	96 rxns
2X SYBR® RT Buffer	25 µL	2.64 mL
20X RT Enzyme Mix*	2.5 µL	264 µL
Nuclease-free Water	12.5 µL	1.32 mL
Final volume RT master mix	40 µL	4.22 mL

* For the minus-RT control, use Nuclease-free Water in place of 20X RT Enzyme Mix.

b. Mix gently, but thoroughly, centrifuge briefly, then place the RT Master Mix on ice.

c. Distribute RT Master Mix to nuclease-free PCR tubes or wells of a multiwell plate.

3. Add lysate and mix thoroughly

a. Add sample lysate to each aliquot of RT Master Mix for a final 50 µL reaction volume.

b. Once assembled, mix reactions gently, then centrifuge briefly to collect the contents at the bottom of the reaction vessel.


STOPPING POINT

Assembled RT reactions can be stored at 4°C for up to 4 hr.

4. Run the RT thermal cycler program

Using a thermal cycler (or real-time PCR instrument), incubate at 37°C for 60 min, then at 95°C for 5 min to inactivate the RT enzyme.


STOPPING POINT

Completed RT reactions may be stored at –20°C.

D. Fast Real-Time PCR

1. Program the real-time PCR instrument

Program the fast-capable real-time PCR instrument as shown in Table 5. Specify SYBR Green fluorescent dye for the experiment.

IMPORTANT

Select "Fast mode" on the Applied Biosystems real-time PCR Instrument.

IMPORTANT

Fast SYBR Green PCR Master Mix contains ROX™ passive reference dye.

Table 5. Real-Time PCR Cycling Conditions

	Stage	Reps	Temp	Time
Enzyme Activation (hold)	1	1	95°C	20 sec
PCR (cycle)	2	40	95°C	3 sec
			60°C	30 sec
Dissociation Curve	3	(use default setting)		

2. Assemble *PCR Cocktail* and distribute to reaction tubes/plates

a. Calculate the number of PCR assays in the experiment. Using Table 6 below, assemble a ***PCR Cocktail*** for all the reactions plus ~10% overage in a nuclease-free microcentrifuge tube at room temperature. Table 6 shows the reaction setup using the recommended amount of cDNA in each PCR, 20%. Up to 30% of the PCR volume can be cDNA; adjust the quantity of Nuclease-free Water accordingly.

NOTE

Before use, mix the Fast SYBR Green PCR Master Mix by swirling the bottle. Mix PCR primers by vortexing briefly or flicking the tube a few times, and then centrifuging.

Table 6. PCR Cocktail Setup

Component	20 µL PCRs Each rxn
Fast SYBR® Green PCR Master Mix	10 µL
Forward & Reverse PCR Primers*	variable
Nuclease-free water	variable
final volume PCR cocktail	16 µL

* Generally a 200–400 nM final concentration of each PCR primer provides good results. For instructions on optimizing PCR primer concentrations, see the Applied Biosystems Fast SYBR® Green PCR Master Mix Protocol, P/N 4385372.

b. Distribute the PCR Cocktail into individual PCR tubes or wells of a real-time PCR plate at room temp.

IMPORTANT

Be sure to use reaction containers and lids that are compatible with your real-time PCR instrument.

3. Add cDNA samples and mix thoroughly

a. Add a portion of the RT reaction (cDNA sample) to each aliquot of PCR Cocktail as indicated in Table 7.

Table 7. PCR Setup

Component	Volume
PCR Cocktail	16 µL
RT Reaction (cDNA)	4 µL

b. Cover the plate or close the tubes, and mix gently. Then centrifuge briefly to remove bubbles and collect the contents at the bottom of the wells/tubes.

4. Run the PCRs in a fast-capable real-time PCR instrument

Place the reactions in a real-time PCR instrument capable of fast cycling and start the run using the settings programmed in step 1 on page 12.

Refer to your real-time PCR instrument guide for information on evaluating the data.

III. Troubleshooting

A. No PCR Product or Unexpected PCR Products

Problems with adding or mixing the Stop Solution

Components in the Lysis Solution may inhibit RT-PCR if they are not fully inactivated by the Stop Solution.

- Be sure to add the Stop Solution directly to the lysate: touch the lysate with the opening of the pipette tip when adding the Stop Solution to make sure that the entire 5 or 6 µL of Stop Solution is added to each sample.
- Mix by pipetting up and down five times.

RNA was degraded before starting the procedure

To avoid RNA degradation, keep cells in PBS on ice before starting the cell lysis procedure. Take cells off ice just prior to adding Lysis Solution.

RNase in the sample was not completely inactivated

Too many cells were used in the lysis reaction

If too many cells per sample are used in the procedure, the RNase in the sample may not be totally inactivated and/or cellular components or debris could inhibit reverse transcription or PCR.

- Generally ≤10^5 cells can be used successfully in the Cells-to-CT™ procedure, but if RT or PCR fails, try using 5- to 10-fold fewer cells.
- Consider performing a pilot experiment to determine the optimal number of cells for your cell type (section IV.B on page 18).
- See "PCR Primer Design Suggestions" on page 17. Suboptimal primer design may become apparent at higher cell inputs.

Too much PBS was left on the cells, diluting the Lysis Solution

If >5 µL of PBS remains in samples when the Lysis Solution is added, the Lysis Solution may be too dilute to fully inactivate cellular RNases. Remove as much PBS as possible before adding Lysis Solution to the cells, or if you split the cells after the PBS wash, resuspend cells in ≤5 µL PBS for each sample of 10–10^5 cells.

Lysates sat too long before going into RT

Do not allow lysates to sit longer than 20 min at room temperature once the Stop Solution has been added: either freeze the lysates at –20°C or –80°C, or start the RT reactions. Alternatively, lysates can be safely stored on ice for up to 2 hr after lysis.

The sample does not contain the target RNA

Negative results are often difficult to confirm as valid. Consider running the following experiments before concluding that the sample does not contain the RNA of interest:

- ***Verify that the procedure is working*** by including Xeno™ RNA Control (from the SYBR Green Cells-to-CT Control Kit, sold separately) in the sample in step II.B.5.a on page 10. Then use the Xeno™

RNA Control PCR primer set to amplify a Xeno RNA target following the instructions in section II.D on page 12. If product is generated in the Xeno RNA amplification, but no product is seen in the PCR for the gene of interest, then it is possible that the RNA of interest is not expressed in the cells and/or is undetectable with this procedure.

- ***For experiments with samples consisting of <100 cells per lysis,*** it may be desirable to verify that each sample did, in fact, contain cells. Check that samples contained cells with intact RNA by real-time RT-PCR with PCR primers for a highly-expressed endogenous control, such as β-actin. The ACTB primers included in the SYBR Green Cells-to-CT Control Kit (sold separately) are designed for this purpose.
- ***Check that the PCR for your target works with your PCR primers, reagents, and equipment*** by using cDNA generated from purified RNA from the same source (or a similar one) in PCR. If the amplification does not give good results using cDNA from purified RNA, it will not work with Cells-to-CT lysate. (See section IV.A on page 17 of this protocol for information on PCR primer design. "Optimizing Primer Concentrations" in the Fast SYBR Green Master Mix Protocol, P/N 4385372, may also provide useful information for optimizing SYBR Green real-time PCR.)

Inhibitors in the RT reaction or PCR

To check for the presence of RT or PCR inhibitors in the Cells-to-CT lysate, purify the RNA from the lysate using a standard method, then compare RT-PCR results from the purified RNA to that from the lysate.

See section *IV.C. Purification of RNA from Cells-to-CT Lysates* starting on page 20.

B. RT-PCR Products in the Negative Control Reactions

PCR products in the no-template PCR control

PCR products in the no-template PCR control most commonly indicate that this sample is contaminated with DNA—typically from completed PCRs. Contamination of PCR reagents, pipettors, and benchtops with DNA should be considered.

- Careful laboratory practices are essential to avoid contaminating reactions with PCR products. Keep concentrated DNA solutions (for example, PCR products, plasmid preps) away from the area where PCRs are assembled. Clean the lab bench and the pipettors routinely with Ambion DNA*Zap*™ Solution (P/N AM9890) or another DNA decontamination product. Use barrier tips to pipette PCR reagents, and store completed PCRs in a different location from the PCR reagents. Unfortunately, the only way to remedy contaminated reagents is to replace them.

- It is always a good idea to routinely include a no-template negative control reaction with experimental PCRs. If no-template controls routinely yield PCR products, more stringent steps may be taken to control contamination.

Primer-Dimer

Primer-dimer is nonspecific PCR product generated from extension of self-annealed PCR primers. In the absence of template or in reactions with poorly designed PCR primers, primer-dimer may be the main reaction product.

Check the dissociation curves of minus-template control reactions to determine whether products are due to contamination from previous PCRs or primer-dimer formation. Primer-dimer products are shorter than the expected amplicon, and thus will have a lower T_m.

PCR products in the minus-RT control

If PCR products are seen in the minus-RT control reaction, but not in the no-template control, it indicates that genomic DNA remains in the samples and that genomic DNA was amplified in real-time PCR. Consider the following suggestions:

- Be sure to mix DNase I into the Lysis Solution thoroughly in step II.B.2 on page 9 for efficient removal of genomic DNA from samples.
- Consider using PCR primers designed to span an exon-exon boundary; amplicons from genomic DNA would thus be too long for efficient PCR amplification.
- Use fewer cells per lysis reaction.
- Lyse cells (step II.B.3 on page 10) using Lysis Solution that is at room temperature, and make sure that lysis reactions occur at room temperature (19–25°C).

If PCR products are still seen in minus-RT control reactions, try the following two suggestions in the order shown:

- Increase the lysis reaction incubation time in step II.B.4 on page 10 to 8 minutes.
- Use Lysis Solution that has been warmed to 25°C for cell lysis (step II.B.3 on page 10).

IV. Appendix

A. PCR Primer Design Suggestions

Identifying target sequence and amplicon size

In this protocol, the target sequence is defined as the DNA sequence (i.e., cDNA, genomic DNA, or plasmid DNA) that you want to amplify. PCR primers are designed to amplify *amplicons* (segments of DNA) within the target sequence (for example using Applied Biosystems Primer Express Software, P/N 4330710). Good amplicon sites will result in amplification of cDNA from the target mRNA without co-amplification of its genomic sequence, pseudogenes, or cDNA from related genes. Following are general guidelines for choosing amplicon sites:

- Shorter amplicons work best. Consistent results are obtained for amplicon size ranges from 50 to 150 bp.
- The amplicon should span one or more introns to avoid amplification of the target gene in genomic DNA.
- The primer pair has to be specific to the target gene; it should not amplify pseudogenes or other related genes.
- Design primers following Primer Express Software guidelines.
- Test the amplicons and select ones that have the highest signal-to-noise ratio (i.e., low C_T with cDNA and no amplification in minus-RT or no-template controls).
- If the gene of interest does not have introns, then it will not be possible to choose an amplicon that will discriminate between cDNA and genomic DNA templates. For such targets, it is a good idea to include minus-RT controls.

Designing PCR primers

Using Primer Express® Software

Design primers using Primer Express Software as described in the *Primer Express® Version 3.0 Getting Started Guide* (P/N 4362460) and Online Help.

General Guidelines

- Keep the T_m between 59–60°C.
- The optimal primer length is 20 nt.
- Keep the GC content in the range of 35–65%.
- Avoid runs of identical nucleotides. If repeats are unavoidable, make sure there are fewer than four consecutive G residues.
- Include no more than two G and/or C bases in the last five nucleotides at the 3' end.
- Avoid amplicons of 150 bp or more.

B. Pilot Experiment

The purpose of this pilot experiment is to identify the maximum number of cells to use in Fast SYBR® Green Cells-to-CT™ reactions. Using too many cells can result in inefficient cell lysis and RT-PCR inhibition, and the maximum number of cells varies somewhat according to the cell type.

In this simple experiment, cells are serially diluted and lysed following the normal protocol. If you have the SYBR® Green Cells-to-CT Control Kit (P/N 4402959), we recommend adding Xeno™ RNA Control to the Stop Solution used to prepare Cells-to-CT lysates. The lysates are then subjected to real-time RT-PCR for an endogenous control gene, such as β-actin (a set of PCR primers for β-actin is included in the SYBR Green Cells-to-CT Control Kit). The C_T values are plotted against the log of the number of cells in the lysis reaction. The resulting line is linear for cell numbers that are compatible with the procedure and deviates from linearity at concentrations that result in incomplete lysis or RT-PCR inhibition. The cDNA can be amplified in parallel using the Xeno RNA PCR primer set (from the SYBR Green Cells-to-CT Control Kit). In contrast to the series of PCRs for an endogenous control gene, the number of cells in the lysis reaction should have no effect on the C_T value seen in Xeno RNA amplification reactions, because each reaction contains the same amount of Xeno RNA target.

1. Harvest, count, and wash cells

Follow the instructions for harvesting, counting, and washing cells in step II.B.1 on page 9, but follow the instructions in step 2 (next) for resuspending them in PBS.

2. Dilute cells to 2 x 10^5 cells/µL

Prepare a cell suspension containing 2 x 10^5 cells/µL. Keep the cell suspension on ice.

3. Make 5 serial dilutions of the cells in 5-fold increments

a. Prepare 5 tubes containing 45 µL of cold 1X PBS in ice.

b. Transfer 5 µL of the 2 x 10^5 cells/µL to the first tube (1:5 dilution) and mix gently but thoroughly. Continue making the serial dilutions by transferring 5 µL of each solution to the subsequent tube to finish with 5 suspensions containing 2 x 10^4, 2000, 200, 20, and 2 cells per µL.

4. Transfer cells to reaction containers with 3 replicates

Transfer 5 µL of each cell suspension to individual reaction tubes or wells of a multiwell plate. Include 3 biological replicates of each cell concentration.

The final cell counts will be 10^5, 10^4, 1000, 100, and 10 cells.

5. Lyse cells in 50 µL of Lysis Solution and incubate at room temp for 5 min

a. Add 50 µL of Cells-to-CT Lysis Solution to each of the samples prepared in step 4.

b. Mix thoroughly by pipetting up and down 5 times.

c. Incubate at room temperature for 5 min.

6. Add 5–6 µL Stop Solution, mix, and incubate at room temp for 2 min

a. (Optional) Add Xeno RNA Control to the Stop Solution.
Mix 16.5 µL Xeno RNA Control with 82.5 µL Stop Solution for use in the next step.

b. Add 5 µL Stop Solution alone or 6 µL Stop Solution with Xeno RNA Control to each cell lysate, and mix thoroughly by pipetting up and down 5 times.

c. Incubate at room temp for 2 min.

7. Perform RT-PCR

Follow the protocols described in sections II.C–D to perform reverse transcription and PCR using all samples, using primers for the following key targets.

- Amplify any endogenous control target, for example using the β-actin PCR primer set included in the SYBR Green Cells-to-CT Control Kit.
- If Xeno RNA Control was added to samples, set up a separate PCR using the Xeno RNA PCR primer set included in the SYBR Green Cells-to-CT Control Kit.
- Use PCR primers for the target of interest and evaluate real-time PCR of the cell titration, to help determine the minimum number of cells required for its detection.

8. Evaluate results

Figure 3 on page 20 displays representative pilot experiment results, discussed below.

Endogenous control

Create a plot of C_T versus the log of the number of cells in the lysis. The C_T values should decrease in a linear fashion as the number of cells increase, for cell numbers that are compatible with the procedure. When the number of cells per lysis reaction exceeds the capacity of the system, resulting in incomplete lysis or inhibition of RT-PCR, the data is not linear. In future experiments, do not exceed the number of cells per lysis reaction that provided results within the linear range in the pilot experiment.

Xeno™ RNA Control

The C_T values from the Xeno RNA Control should be consistent (±1 C_T), regardless of the number of cells in the lysis reaction, indicating that no RT-PCR inhibitors are present in the Cells-to-CT lysate. Higher C_T values at higher numbers of cells per lysis reaction indicate that inhibitors were introduced into RT-PCR with this number of cells. For future experiments, use only the number of cells per lysis reaction that did not show an increase in C_T value.

Target of interest

The pilot experiment can provide useful information about the number of cells required to detect the target of interest. Examine the results carefully and choose cell numbers that will provide sufficient signal for the experiment.

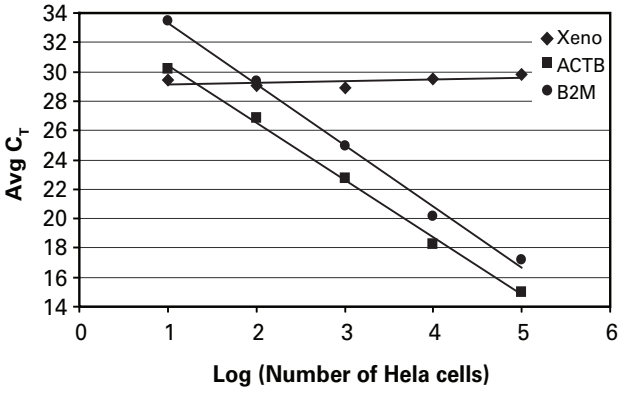


Figure 3. Example Pilot Experiment

HeLa cells were diluted and lysed following the instructions for the pilot experiment. Lysates were reverse transcribed and amplified using the indicated primer pairs in duplicate reactions. The resulting C_T values were plotted against the log of the number of cells used in the lysis reaction (as recommended). This experiment shows that even at 10^5 cells per lysis reaction, RT-PCR is not compromised by reaction inhibitors carried over from the cell lysate. Xeno: Xeno™ RNA; ACTB: beta-actin; B2M: beta 2-Microglobulin.

C. Purification of RNA from Cells-to-CT Lysates

If purification of RNA from Cells-to-CT lysates is required for your experimental needs, follow the manufacturers' recommended protocol using the cell lysate from step II.B.6 on page 10 as the starting sample.

For example, to use the Ambion RNAqueous®-4PCR Kit (P/N AM1914), start the purification at step 2 in "RNA Isolation Procedure" of the RNAqueous®-4PCR Kit protocol (P/N 1914M):

1. In step 2, add 300 µL RNAqueous-4PCR Lysis/Binding Solution to the Cells-to-CT lysate (55 µL) and vortex.
   For cells lysed in a 96-well plate, first add 100 µL of Lysis/Binding Solution to each well and mix by pipetting up and down. Transfer to a nuclease-free microcentrifuge tube. Add an additional 200 µL of Lysis/Binding Solution and vortex to mix.
2. Continue the RNAqueous-4PCR protocol at step 3 (add an equal volume [355 µL] of 64% ethanol).

D. Quality Control

To obtain a Certificate of Analysis for this kit, go to http://www3.appliedbiosystems.com/sup/coa/search.htm and enter the part number/catalog # and batch number/lot #.

E. Safety Information

WARNING

GENERAL SAFETY. *Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.*

- *Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.*
- *Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.*

1. Chemical safety

WARNING

GENERAL CHEMICAL HANDLING. *To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:*

- *Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.*
- *Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).*
- *Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).*
- *Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.*
- *Handle chemical wastes in a fume hood.*
- *Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)*
- *After emptying a waste container, seal it with the cap provided.*

- *Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.*
- *Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.*
- ***IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.*

2. Biological hazard safety

WARNING

***Potential Biohazard.** Depending on the samples used on the instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.*

WARNING

***BIOHAZARD.** Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:*

In the U.S.:

- *U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: www.cdc.gov/biosafety*
- *Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html*
- *Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.*
- *Additional information about biohazard guidelines is available at: www.cdc.gov*

In the EU:

Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/

V. Documentation and Support

A. Obtaining SDSs

Safety Data Sheets (SDSs) are available from:

www.invitrogen.com/sds

or

www.appliedbiosystems.com/sds

Note: For the SDSs of chemicals not distributed by Life Technologies, **contact the chemical manufacturer.**

B. Obtaining support

For the latest services and support information for all locations, go to:

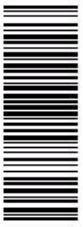
www.invitrogen.com

or

www.appliedbiosystems.com

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches



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