TaqMan® PreAmp Cells-to-CT™ Kit

PreAmplified Two-Step RT-PCR Directly from Cultured Cell Lysates

Part Number 4387299

TaqMan® PreAmp Cells-to-CT™ Kit

(P/N 4387299)

Protocol

P/N 4387854 Revision D **Revision Date: August 23, 2010**

For research use only. Not for use in diagnostic procedures. By use of this product, you accept the terms and conditions of all applicable Limited Label Licenses. For statement(s) and/or disclaimer(s) applicable to this product, see below.

Information in this document is subject to change without notice. Applied Biosystems assumes no responsibility for any errors that may appear in this document.

Applied Biosystems disclaims all warranties with respect to this document, expressed or implied, including but not limited to those of merchantability or fitness for a particular purpose. In no event shall Applied Biosystems be liable, whether in contract, tort, warranty, or under any statute or on any other basis for special, incidental, indirect, punitive, multiple or consequential damages in connection with or arising from this document, including but not limited to the use thereof.

Literature Citation When describing a procedure using this product for publication, please refer to it as the TaqMan® PreAmp Cells-to-CT™ Kit. Author(s) of research journal publications that cite Ambion products may receive a complimentary T-shirt by sending in the completed form at the back of this protocol, along with a copy of the paper.

Warranty and Liability Applied Biosystems is committed to delivering superior product quality and performance, supported by industry-leading global service and technical support teams. Warranty information for the accompanying consumable product is available at www.ambion.com/info/warranty in "Limited Warranty for Consumables," which is subject to the exclusions, conditions, exceptions, and limitations set forth under the caption "EXCLUSIONS, CONDITIONS, EXCEPTIONS, AND LIMITATIONS" in the full warranty statement. Please contact Applied Biosystems if you have any questions about our warranties or would like information about post-warranty support.

Notice to Purchaser: Limited License A license to perform the patented 5' Nuclease Process for research is obtained by the purchase of (i) both Authorized 5' Nuclease Core Kit and Licensed Probe, (ii) a Licensed 5' Nuclease Kit, or (iii) license rights from Applied Biosystems.

This product is an Authorized 5' Nuclease Core Kit. Use of this product is covered by US patent claims and patent claims outside the US. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. Separate purchase of a Licensed Probe would convey rights under the applicable claims of US Patents, and claims outside the United States. No right under any other patent claim and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses under Roche patents require a separate license from Roche. Further information on purchasing licenses may be obtained from the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

Trademarks Applied Biosystems, AB (Design), Ambion, GeneAmp, MicroAmp and RNase*Zap* are registered trademarks, and Cells-to-CT, DNA*Zap,* MultiScribe, ROX, StepOne, StepOnePlus, Veriti, FAM and XenoRNA are trademarks of Applera Corporation or its subsidiaries in the US and/or certain other countries. TaqMan is a registered trademark of Roche Molecular Systems, Inc. All other trademarks are the sole property of their respective owners.

I. Introduction

A. Product Description and Background

Reverse transcription (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 can lead 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.

The TaqMan® PreAmp Cells-to-CT™ Kit includes Applied Biosystems TaqMan PreAmp Master Mix for preamplification of up to 100 specific targets. TaqMan Gene Expression Assays for the targets of interest are diluted and used in a 10 or 14 cycle preamplification reaction to provide unbiased amplification of the specific amplicons. Preamplification enriches cDNA for the selected targets, providing more material for subsequent real-time PCR. This is beneficial when target quantity is limited and/or when a single sample will be used for many downstream real-time PCRs.

Cells-to-CT lysates exhibit sensitivity and specificity similar to that from purified RNA in real-time RT-PCR using TaqMan® Gene Expression Assays. 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 TaqMan PreAmp 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 RT Buffer.
- Specific targets of interest in the cDNA are then preamplified for 10 or 14 thermal cycles using a pool of up to 100 diluted TaqMan Gene Expression Assays and the included TaqMan PreAmp Master Mix.
- Finally, the diluted preamplification product is amplified by real-time PCR using the included TaqMan Gene Expression Master Mix and the TaqMan Gene Expression Assay for your target-of-interest (assays sold separately).

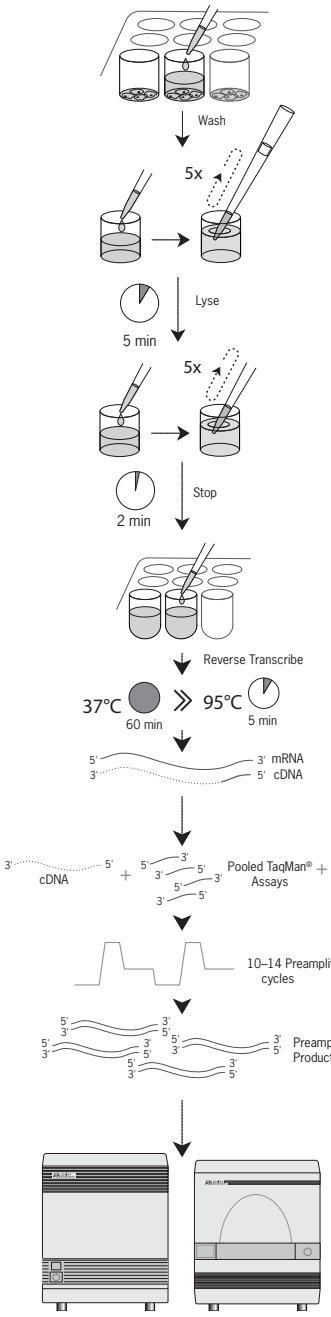
TaqMan® Cells-to-CT™ Control Kit

The TaqMan Cells-to-CT Control Kit (P/N 4386995, available separately) is designed for use with the TaqMan Gene Expression, TaqMan PreAmp, and TaqMan Fast Cells-to-CT Kits. It includes XenoRNA™ Control, an RNA transcript that has no homology to any known sequences, and a XenoRNA TaqMan Gene Expression Assay to detect the XenoRNA Control target. It also includes a TaqMan Assay for the highly expressed endogenous control gene, β-actin. These assays are designed to be run in parallel (not in multiplex PCR) with assay(s) for the gene(s)-of-interest, to serve as positive controls for RT and real-time PCR.

Applications

The TaqMan® PreAmp Cells-to-CT™ Kit can be used in any real-time RT-PCR application to analyze mRNA from cultured cells. It is particularly useful for limited samples or applications where large numbers of PCRs will be run from each sample. The development process included extensive testing for sensitivity and specificity with a broad selection of TaqMan Gene Expression Assays. The kit is well suited for large experiments, such as real-time 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 1. TaqMan® PreAmp Cells-to-CT™ Procedure Overview



Cell Lysis

1. Wash cells in cold PBS in the culture plate, or wash and transfer ≤10^5 cells to tubes or plates for lysis
2. (Optional) Dilute DNase I into Lysis Solution at 1:100
3. Add 50 µL Lysis Solution and mix 5 times
4. Incubate for 5 min at room temp (19–25°C)
5. Add 5 µL Stop Solution, or 6 µL Stop Solution with XenoRNA™ Control, and mix 5 times
6. Incubate for 2 min at room temp

Potential stopping point

Reverse Transcription (RT)

1. Program the thermal cycler for the RT
2. Assemble an RT Master Mix and distribute to reaction tubes/plates
3. Add lysate and mix thoroughly
4. Run the RT thermal cycler program

Potential stopping point

Preamplification

1. Pool the TaqMan Assays for your targets-of-interest
2. Program the thermal cycler
3. Assemble the preamplification reactions and mix thoroughly
4. Run the preamplification in a thermal cycler then immediately place the reactions on ice

Potential stopping point

5. Dilute the preamplification products

Real-Time PCR

1. Program the real-time PCR instrument
2. Assemble PCR Cocktail and aliquot into reaction tubes/plates
3. Add diluted PreAmp product and mix thoroughly
4. Run the PCRs in a real-time PCR instrument

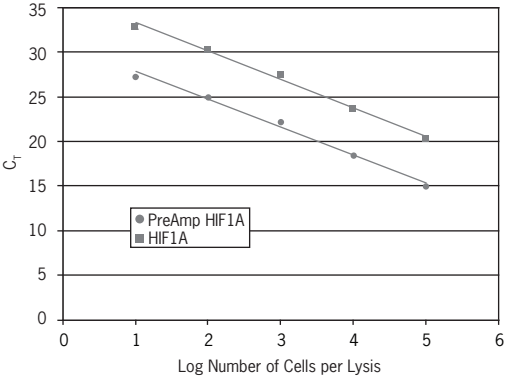


Figure 2. Real-time RT-PCR Using the TaqMan® PreAmp Cells-to-CT™ Kit

A dilution series of 10–10^5 HeLa cells was processed in triplicate with the TaqMan® PreAmp Cells-to-CT™ Kit. The gene HIF1A (Assay ID Hs00153153_m1) was amplified from cDNA (not preamplified) and diluted preamplification product in triplicate. For both templates, amplification was linear over a cell input range of 10–10^5 cells per lysis. Note that preamplification caused a reduction of ~5 C_T values in real-time PCR.

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

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

Cell Line	Growth	Source Species	Source Tissue
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

Reagents for 40 lysis reactions with genomic DNA removal are provided in the TaqMan PreAmp Cells-to-CT Kit. For each lysis reaction, reagents for one reverse transcription (RT) reaction plus one minus-RT control are also provided, as well as master mix for one preamplification reaction. The Gene Expression Master Mix can be used for either 200, 50 µL real-time PCR assays or 500, 20 µL reactions. The kit does ***not*** include TaqMan Gene Expression Assays, which are needed for preamplification and real-time PCR for your target(s) of interest.

Amount	Component	Storage
200 µL	Stop Solution	–20°C
22 µL	DNase I	–20°C
110 µL	20X RT Enzyme Mix	–20°C
2.2 mL	Lysis Solution	4°C
2.2 mL	2X RT Buffer	4°C
1 mL	TaqMan® PreAmp Master Mix	4°C
5 mL	TaqMan® Gene Expression Master Mix	4°C

NOTE

The product is shipped on dry ice, but once received, 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)
- Real-time PCR tubes or 96-well plates appropriate for your instrument

- Thermal cycling instrument: The TaqMan 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 any real-time PCR system compatible with the fluorophores used in TaqMan Gene Expression Assays. TaqMan PreAmp 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 (in "standard" mode)
  - Applied Biosystems 7500 Real-Time PCR System
  - StepOne™ or StepOnePlus™ Real-Time PCR Systems

Reagents

- RT-PCR grade water, for example Ambion P/N AM9935
- Phosphate-buffered saline (PBS), for example diluted from Ambion 10X PBS, P/N AM9624 and AM9625
- Molecular biology grade TE buffer: 10 mM Tris-HCl (pH ~8), 1 mM EDTA, for example Ambion TE, pH 8.0 (P/N AM9849)
- TaqMan® Gene Expression Assay for real-time PCR of the target(s) of interest (Applied Biosystems P/N 4331182, 4351372).
- (optional) TaqMan® Cells-to-Ct™ Control Kit (P/N 4386995)

D. Related Products Available from Applied Biosystems

TaqMan® Cells-to-Ct™ Control Kit P/N 4386995	Designed for use with TaqMan Cells-to-Ct Kits, the Control Kit includes XenoRNA™ Control, an RNA transcript that has no homology to any known sequence, and a corresponding XenoRNA TaqMan Gene Expression Assay. It also includes a TaqMan Assay for the highly expressed endogenous control gene β-Actin. These assays are designed to be run in parallel with assay(s) for the gene(s)-of-interest to serve as positive controls for reverse transcription and real-time PCR.
TaqMan® Gene Expression Assays P/N 4331182 P/N 4351372	Pre-designed and pre-optimized TaqMan® probe and primer sets for quantitative gene expression analysis using real-time PCR. Each TaqMan Assay is a ready-to-use 20X mixture of PCR primers and TaqMan probe designed for amplification using universal cycling conditions so that any combination of assays can be run using the same thermal cycling conditions.There are over 700,000 inventoried assays for human, mouse, rat, *Arabidopsis*, *Drosophila*, *C. elegans*, *C. familiaris* (dog), and *Rhesus macaque* genes, and Custom TaqMan Gene Expression Assays are also available.

Applied Biosystems Real-Time PCR Instruments See web or print catalog for P/Ns	Since pioneering real-time PCR, Applied Biosystems has continued to develop the technology to provide more powerful solutions for labs of all sizes. The Applied Biosystems family of real-time platforms, which includes the Applied Biosystems 7300, 7500, 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.
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 will eliminate all traces of RNase and RNase*Zap* Solution.
RNase-free Tubes & Tips See web or print 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. See our latest catalog or our website (www.ambion.com/prod/tubes) for specific information.
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 web or print 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 high levels of contaminating DNA and RNA from surfaces instantly.

II. TaqMan® PreAmp Cells-to-CT™ Procedure

A. Suggested Controls and Replicates

Biological and technical replicates

The definitions of biological and technical replicates can have different interpretations; here we define biological replicates as separate lysis reactions and technical replicates as separate PCRs. Our 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); they demonstrate that the template for the PCR was cDNA, and not genomic DNA.

NOTE

Minus-RT controls may not be necessary if the cDNA will be amplified using a TaqMan® Gene Expression Assay with the suffix "_m"; these assays are designed to span an exon-exon junction and will not amplify genomic DNA.*

No-template control

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

Preamplification uniformity

Before performing preamplification with limited biological samples, we recommend checking whether all amplicons are amplified without bias using the experiment described in section IV.B starting on page 22.

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 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 tubes or plates 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.A starting on page 19. 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 into Lysis Solution at 1:100

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	Amount per reaction
Lysis Solution	49.5 µL
DNase I	0.5 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, or 6 µL Stop Solution with XenoRNA™ Control, and mix 5 times

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

b. Pipet 5 µL of Stop Solution alone or 6 µL of Stop Solution with XenoRNA Control directly into each lysis reaction.
Do this by touching the surface of the lysate with the opening of the pipet tip to ensure that all of the Stop Solution is added to the lysate.


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, unused Stop Solution can be stored frozen in these tubes when properly sealed.

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.


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 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. If desired, 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 PCR assays, the RT reaction can be scaled up proportionally.

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

Component	Amount per reaction
2X RT Buffer	25 µL
20X RT Enzyme Mix*	2.5 µL
Nuclease-free Water	12.5 µL
final volume RT master mix	40 µL

* 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. Preamplification

Preamplification using this kit is essentially a truncated multiplex PCR of up to 100 specific cDNA targets used to increase their abundance for subsequent gene expression analysis using TaqMan Gene Expression Assays.

Recommendations for preamplification

- Pool TaqMan Gene Expression Assays that result in a $C_T \leq 35$ when used in an amplification reaction using your experimental parameters. Note that preamplification of targets which result in C_T values >35 is not recommended because subsequent amplification may not be linear.
- ***Do not*** include the 18S TaqMan Gene Expression Assay in the pool due to the high expression level of this gene. ***Do*** include other control assays, such as the ACTB and XenoRNA Gene Expression Assays included with the TaqMan Cells-to-CT Control Kit.
- Before performing preamplification with limited biological samples, we recommend checking whether all amplicons are amplified without bias using the experiment described in section IV.B starting on page 22.

1. Pool the TaqMan Assays for your targets-of-interest

IMPORTANT

Keep all TaqMan Gene Expression Assays in the freezer, protected from light, until you are ready to use them. Excessive exposure to light may affect the performance of the fluorescent probes.

a. Thaw the TaqMan Gene Expression Assays. When thawed, mix the assays by vortexing and then centrifuge the tubes briefly.

b. In a microcentrifuge tube, combine equal volumes of each 20X TaqMan Gene Expression Assay, up to a total of 100 assays. For example, to pool 50 assays, combine 10 µL of each assay.

c. Dilute the pooled TaqMan Gene Expression Assays using 1X TE buffer so that each assay is at a final concentration of 0.2X. For the above example, add 500 µL 1X TE buffer to the pooled TaqMan assays for a total volume of 1 mL.

2. Program the thermal cycler

a. Determine the appropriate number of preamplification cycles:
   - ***10 cycles:*** Recommended for preamplification with small numbers of pooled assays or when ≤250 µL preamplification product is enough for the subsequent PCRs planned. The 10 cycle preamplification produces enough material for fifty 20 µL PCR assays or twenty 50 µL PCR assays.

- ***14 cycles:*** Recommended for preamplification with higher numbers of pooled assays or when 1 mL of preamplification product is needed for the subsequent PCRs. The 14 cycle preamplification produces enough material for two hundred 20 µL PCR assays or eighty 50 µL PCR assays.

b. Program the thermal cycler to run the PCR profile shown in Table 5.

Table 5. Preamplification PCR Cycling Conditions

	Stage	Reps	Temp	Time
Enzyme activation (hold)	1	1	95°C	10 min
PCR (cycle)	2	10 or 14	95°C	15 sec
			60°C	4 min

3. Assemble the preamplification reactions and mix thoroughly

a. At room temp, assemble the reagents shown in Table 6 in a tube or plate suitable for the thermal cycler.

NOTE

Before use, mix the TaqMan PreAmp Master Mix by inverting the tube and briefly centrifuging.

Table 6. Preamplification Reaction Setup

Component	Amount
TaqMan PreAmp Master Mix	25 µL
Pooled assay mix (0.2X)	12.5 µL
cDNA (from the completed RT reaction)	12.5 µL

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

4. Run the preamplification in a thermal cycler then immediately place the reactions on ice

a. Place the preamplification reactions in a thermal cycler and start the run using the settings programmed in step 2 on page 12.

b. Upon completion, ***immediately*** remove the tubes or plate from the thermal cycler and place it on ice.

STOPPING POINT

The preamplification product can be stored at –20°C if desired. We recommend storing it in aliquots to minimize freeze-thaw cycles.

5. Dilute the preamplification products

Dilute the preamplification products with 1X TE, based on the number of preamplification cycles, as shown in Table 7.

Table 7. Dilution of Preamplification Reactions

	10 PreAmp Cycles: Dilute 1:5	14 PreAmp Cycles: Dilute 1:20
PreAmp Reaction	50 µL	50 µL
1X TE	200 µL	950 µL
Final Volume	250 µL	1 mL

E. Real-Time PCR

1. Program the real-time PCR instrument

Program the real-time PCR instrument as in Table 8.

IMPORTANT

On Applied Biosystems real-time PCR instruments capable of Fast mode thermal cycling, select Standard mode.

- TaqMan® Gene Expression Master Mix contains ROX™ passive reference dye.
- Specify the fluorescent dye(s) used in the TaqMan Gene Expression Assays for the experiment.
  The ACTB and XenoRNA™ Gene Expression Assays in the TaqMan Cells-to-CT™ Control Kit are labeled with FAM™ dye and a nonfluorescent quencher.

Table 8. Real-Time PCR Cycling Conditions

	Stage	Reps	Temp	Time
UDG Incubation (hold)	1	1	50°C	2 min
Enzyme Activation (hold)	2	1	95°C	10 min
PCR (cycle)	3	40	95°C	15 sec
			60°C	1 min

2. Assemble *PCR Cocktail* and aliquot into reaction tubes/plates

a. Calculate the number of PCR assays in the experiment. Using Table 9 below, assemble a ***PCR Cocktail*** for all the reactions plus ~10% overage in a nuclease-free microcentrifuge tube at room temp. Table 9 shows the reaction setup using the recommended amount of preamplification product in each PCR, 20%. If desired, up to 45% of the PCR volume can be preamplification product; adjust the quantity of Nuclease-free Water accordingly.

NOTE

Before use, mix the TaqMan Gene Expression Master Mix by swirling the bottle. Mix TaqMan Assays by vortexing briefly or flicking the tube a few times, and then centrifuging.

Table 9. PCR Cocktail Setup

Component	20 µL PCRs Each rxn	50 µL PCRs Each rxn
TaqMan® Gene Expression Master Mix (2X)	10 µL	25 µL
TaqMan® Gene Expression Assay* (20X)	1 µL	2.5 µL
Nuclease-free water	4 µL	10 µL
final volume PCR cocktail	15 µL	37.5 µL

* Not included in the TaqMan PreAmp Cells-to-CT Kit.

IMPORTANT

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

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

3. Add diluted PreAmp product and mix thoroughly

a. Add a portion of the diluted PreAmp reaction products to each aliquot of PCR Cocktail as indicated in Table 10.

Table 10.Final PCR Setup

Component	20 µL PCRs	50 µL PCRs
PCR Cocktail	15 µL	37.5 µL
Diluted PreAmp product (from step D.5 on page 14)	5 µL	12.5 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 real-time PCR instrument

Place the reactions in a real-time PCR instrument and start the run using the settings programmed in step E.1 on page 14.

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, in other words, touch the lysate with the opening of the pipet tip when adding the Stop Solution to make sure that the entire 5 or 6 µL of Stop Solution is added to each sample.
- Also, we recommend mixing 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 fewer cells (e.g., 5–10-fold fewer cells).
- Also, consider performing a pilot experiment to determine the optimal number of cells for your cell type (section IV.A on page 19).

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. To avoid this, 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.

Problems with the preamplification

- With the exception of assays for 18S rRNA, be sure to use the same assays for the preamplification and the real-time PCR. Otherwise levels of gene expression cannot be compared.


NOTE

It is important to exclude 18S assays from the preamplification pool, because the 18S rRNA is so highly expressed that its amplification would deplete the PCR reagents and other targets would not be amplified to any significant degree.

- Preamplification reaction products must be diluted as described in step II.D.5 on page 14 before using them in PCR.

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*** TaqMan® PreAmp Cells-to-CT™ ***procedure is working*** by including XenoRNA™ Control (from the TaqMan Cells-to-CT Control Kit, sold separately) in the sample in step II.B.5.a on page 10. Then use the XenoRNA TaqMan Gene Expression Assay to amplify a XenoRNA target following the instructions in section II.E on page 14. If product is generated in the XenoRNA 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 a TaqMan Gene Expression Assay for a highly-expressed endogenous control such as β-actin using the ACTB assay included in the TaqMan Cells-to-CT Control Kit (sold separately).
- ***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.

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 the sample contains DNA contamination—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 (PCR products, plasmid prep, etc.) 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.

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 a TaqMan Gene Expression Assay designed to span an exon-exon boundary; such assays are designated with _m* at the end of the part number.
- 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 up to 25°C for cell lysis (step II.B.3 on page 10).

IV. Appendix

A. Pilot Experiment

The purpose of this pilot experiment is to identify the maximum number of cells to use in TaqMan® PreAmp 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 TaqMan Cells-to-CT Control Kit (P/N 4386995), we recommend adding XenoRNA™ Control to the Stop Solution used to prepare Cells-to-CT lysates. The lysates are then preamplified and subjected to real-time RT-PCR for an endogenous control gene, such as β-actin, (an ACTB TaqMan Gene Expression Assay is included in the TaqMan Cells-to-CT Control Kit), and the C_T values are plotted against the log of the number of cells in the lysis reaction. The resulting line will be linear for cell numbers that are compatible with the procedure and will deviate from linearity at concentrations that result in incomplete lysis or RT-PCR inhibition. The preamplification product can be amplified in parallel using the XenoRNA TaqMan Gene Expression Assay (from the 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 XenoRNA amplification reactions because each reaction will contain the same amount of XenoRNA target.

1. Harvest, count, and wash cells

Follow the instructions for harvesting 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 XenoRNA Control to the Stop Solution.
Mix 16.5 µL XenoRNA 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 XenoRNA 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–E to reverse transcribe, preamplify, and perform PCR using all samples.

- Preamplify with the same set of diluted TaqMan Gene Expression Assays prepared for the experimental samples.
- Amplify with any Applied Biosystems Endogenous Control TaqMan Gene Expression Assay, for example with the ACTB assay included in the Cells-to-CT Control Kit.
- If XenoRNA Control was added to samples, it can be amplified in a separate PCR using the XenoRNA TaqMan Gene Expression Assay included in the Cells-to-CT Control Kit.
- You can also evaluate real-time PCR of the cell titration using the TaqMan Gene Expression Assay for the target-of-interest to help determine the minimum number of cells required for its detection.

8. Evaluate results

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 will not be 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.

XenoRNA™ Control

The C_T values from the XenoRNA 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. If C_T values begin to increase at higher numbers of cells per lysis reaction, it

indicates 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.

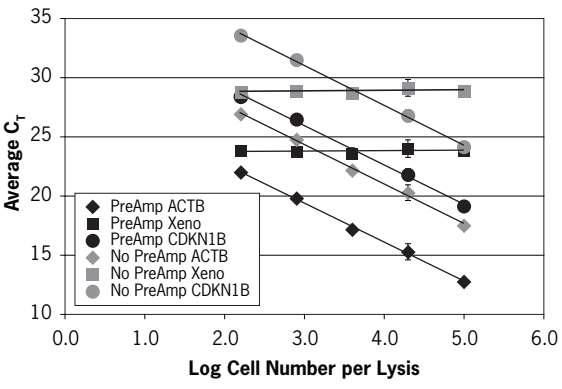


Figure 3. Example Pilot Experiment

HeLa cells were diluted and lysed following the instructions for the pilot experiment. Lysates were reverse transcribed and a portion of the lysates were preamplified using the indicated TaqMan Gene Expression Assays. Both the preamplified products and the not preamplified cDNA were then amplified in three replicate 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. It also shows that preamplification was uniform for these targets and that it resulted in a reduction of the C_T values obtained. Note that it is not necessary to include untreated or not preamplified cDNA in pilot experiment.

B. (Optional) Checking Preamplification Uniformity

To check that preamplification is uniform, in other words, that all targets are amplified without bias, perform this relative quantitation experiment with your selected TaqMan Gene Expression Assays. Using a non-limited, control Cells-to-CT lysate, compare normalized C_T values from preamplified Cells-to-CT lysate to those from lysates that were not preamplified.

1. Prepare Cells-to-CT lysate

Follow the instructions in section II.B starting on page 8 to prepare 50 µL of Cells-to-CT lysate from a non-limited samples, such as a control sample.

2. Reverse transcribe a portion of the lysate

Follow the instructions in section II.C starting on page 11 to reverse transcribe a portion of the lysate. The quantity of lysate used should mimic the conditions you plan to use for experimental samples, in other words, if you plan to use 10 µL of Cells-to-CT lysate in the RT reaction for your experimental samples, use 10 µL of lysate for the RT in this reaction.

3. Preamplify a portion of the cDNA

Follow the instructions in section II.D starting on page 12 to preamplify a portion of the cDNA.

- Include an endogenous uniformity reference gene in your pool. For human gene expression assays, Applied Biosystems recommends using CDKN1B (Assay ID HS00153277_m1) because of its consistent gene expression profile.
- Use the same number of preamplification cycles for this validation experiment as you plan to use for the experimental samples.

4. Run PCRs for preamplified and untreated cDNA

For each TaqMan Gene Expression Assay that was included in the assay pool used for preamplification, assemble sets of PCRs for both the preamplified product and the non-preamplified cDNA. Follow the instructions in section II.E starting on page 14.

5. Evaluate the results

Use a relative quantitation study to analyze your results and to determine $\Delta\Delta C_T$ values between the cDNA that was not preamplified and the preamplified product. Refer to the appropriate relative quantitation document:

- *Applied Biosystems 7900HT Fast Real-Time PCR System Relative Quantitation Using Comparative CT Getting Started Guide (PN 4364016)*
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Relative Quantitation Getting Started Guide (PN 4347824).*

a. Calculate the average C_T values for each assay. [Described in the calculations below as avg $C_{T(\text{target X})}$]

NOTE

Set the non-preamplified cDNA plate as the calibrator under analysis settings.

b. Calculate ΔC_T for untreated cDNA by subtracting the average C_T value of the CDKN1B assays from the average C_T value of each assay:
$\Delta C_{T(cDNA)} = \text{avg } C_{T(\text{target X})} - \text{avg } C_{T(\text{Uniformity of gene})}$

The purpose is to normalize each individual target to the desired uniformity reference gene when using cDNA that is not preamplified. The $\Delta C_{T(cDNA)}$ represents the normalized C_T for the untreated (non-preamplified) cDNA.

c. Calculate ΔC_T for multiplex preamplification by subtracting the average C_T value of the uniformity reference gene from the average C_T value of each assay:
$\Delta C_{T(Preamp)} = \text{avg } C_{T(\text{target X})} - \text{avg } C_{T(\text{Uniformity of gene})}$

The purpose is to normalize each individual target to the desired uniformity reference gene when using preamplified cDNA. The $\Delta C_{T(cDNA)}$ represents the normalized C_T for the preamplified cDNA

d. Calculate the $\Delta\Delta C_T$ for each assay between cDNA and preamplified cDNA by subtracting the ΔC_T value for cDNA (step b) from the ΔC_T for multiplex preamplification (step c):
$\Delta\Delta C_T = \Delta C_{T(Preamp)} - \Delta C_{T(cDNA)}$

A $\Delta\Delta C_T$ value close to zero indicates that preamplification was uniform. Typically, 90% of targets produce $\Delta\Delta C_T$ values that are with 1.5 C_T values of zero. Figure 4 on page 23 shows an example of preamplification uniformity results for 12 assays.

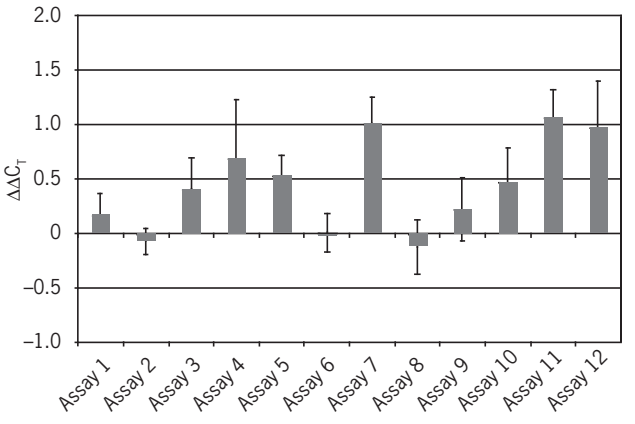


Figure 4. Preamplification is Uniform

An example of a preamplification uniformity experiment is shown here. Note that all 12 assays evaluated showed uniform preamplification ($\Delta\Delta C_T$ within ±1.5).

C. Safety Information

The MSDS for any chemical supplied by Applied Biosystems or Ambion is available to you free 24 hours a day.

IMPORTANT

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

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from product-specific links on our website at the following address: www.ambion.com/techlib/msds
- Alternatively, e-mail your request to: MSDS_Inquiry_CCRM@appliedbiosystems.com. Specify the catalog or part number(s) of the product(s), and we will e-mail the associated MSDSs unless you specify a preference for fax delivery.
- For customers without access to the internet or fax, our technical service department can fulfill MSDS requests placed by telephone or postal mail. (Requests for postal delivery require 1–2 weeks for processing.)

Chemical safety guidelines

To minimize the hazards of chemicals:

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

D. Quality Control

Functional testing

Lysis components are functionally tested by producing cDNA from a serial dilution of 10–10^5 cultured cells, detecting PPIA using real-time PCR, and comparing amplification signal from Cells-to-CT lysates to those obtained from purified RNA from the same source.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with 25 ng labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with 300 ng supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with 5 pmol labeled double-stranded DNA, followed by PAGE analysis.

Protease testing

Meets or exceeds specification when a sample is incubated with 1 µg protease substrate and analyzed by fluorescence.