

USER GUIDE

ambion[®]
by *life* technologies™

TaqMan[®] PreAmp Cells-to-C_T[™] Kit

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TaqMan[®] PreAmp Cells-to-C_T[™] Kit

Introduction

IMPORTANT! Before using this product, read and understand the information the “Safety” appendix in this document.

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-C_T[™] technology enables reverse transcription of lysates from 10–10⁵ 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-C_T[™] 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-C_T[™] 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

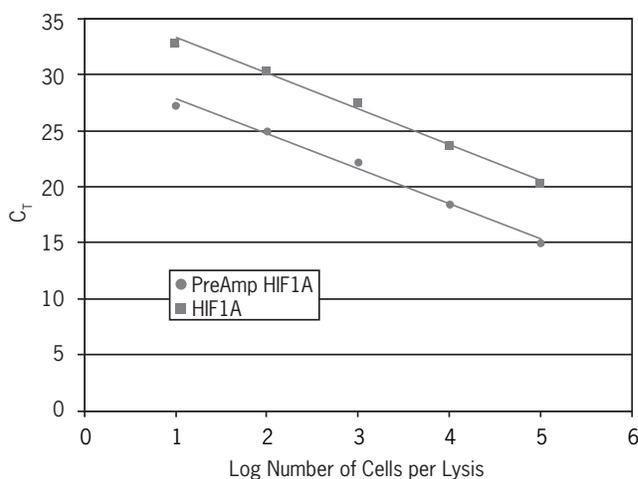
The TaqMan[®] PreAmp Cells-to-C_T[™] Kit procedure is shown in the “Workflow” on page 8 and described below.

- First, 10–10⁵ 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).

Figure 1 Real-time RT-PCR Using the TaqMan® PreAmp Cells-to-C_TTM Kit.

A dilution series of 10–10⁵ HeLa cells was processed in triplicate with the TaqMan® PreAmp Cells-to-C_TTM 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 10–10⁵ cells per lysis. Note that preamplification caused a reduction of ~5 C_T values in real-time PCR.



TaqMan® Cells-to-C_TTM Control Kit

The TaqMan® Cells-to-C_TTM Control Kit (Cat. no. 4386995, available separately) is designed for use with the TaqMan® Gene Expression, TaqMan® PreAmp, and TaqMan® Fast Cells-to-C_TTM Kits. It includes XenoRNATM Control, an RNA transcript that has no homology to any known sequences, and a XenoRNATM TaqMan® Gene Expression Assay to detect the XenoRNATM 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-C_TTM 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.



Cell type compatibility

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

Workflow

Perform cell lysis (page 10)

Wash cells in cold PBS in the culture plate, or wash and transfer $\leq 10^5$ cells to tubes or plates for lysis. (page 10)

(Optional) Dilute DNase I into Lysis Solution at 1:100. (page 11)

Add 50 μ L Lysis Solution and mix 5 times. (page 11)

Incubate the lysis reactions for 5 min at room temp (19–25°C). (page 11)

Add 5 μ L Stop Solution, or 6 μ L Stop Solution with XenoRNA™ Control, and mix 5 times. (page 11)

Incubate for 2 min at room temperature. (page 12)

Potential stopping point

Perform reverse transcription (RT) (page 12)

Program the thermal cycler for RT. (page 12)

Assemble an RT Master Mix and distribute to reaction tubes/plates (page 12)

Add lysate and mix thoroughly. (page 13)

Run the RT thermal cycler program. (page 13)

Preamplification (page 13)

Pool the TaqMan® Assays for your targets-of-interest. (page 13)

Assemble the preamplification reactions and mix thoroughly. (page 14)

Run the preamplification in a thermal cycler then immediately place the reactions on ice. (page 14)

Dilute the preamplification products (page 15)

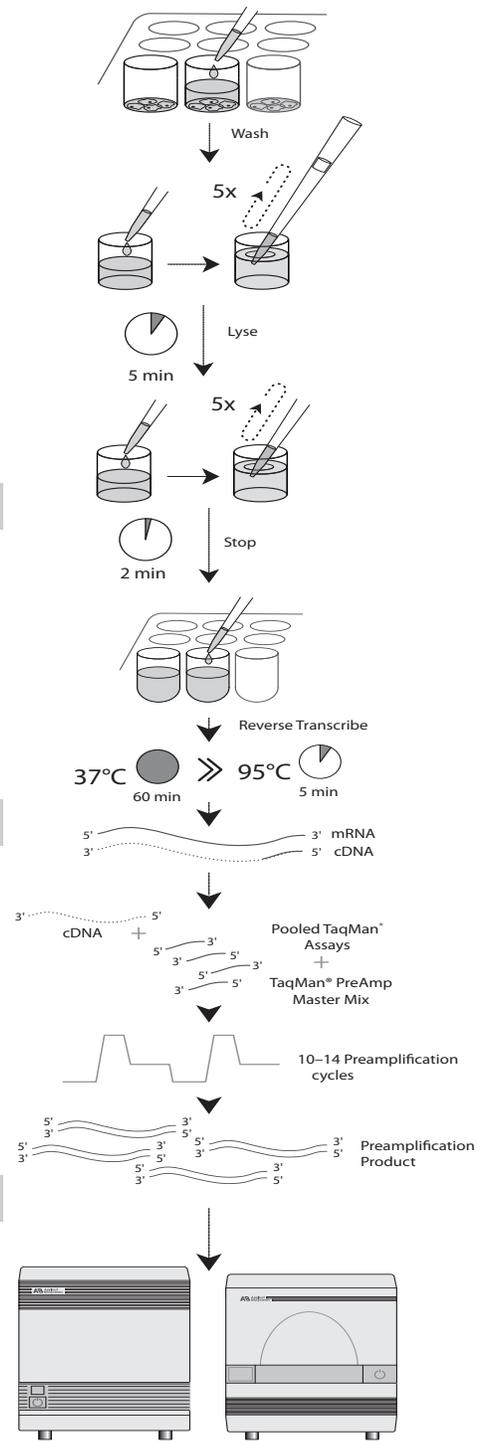
Perform real-time PCR (page 15)

Program the real-time PCR instrument. (page 15)

Assemble the PCR Cocktail and aliquot into reaction tubes/plates. (page 15)

Add diluted PreAmp product and mix thoroughly. (page 16)

Run the PCRs in a real-time PCR instrument. (page 16)





Kit contents and storage conditions

Reagents for 40 lysis reactions with genomic DNA removal are provided in the TaqMan® PreAmp Cells-to-C_T[™] 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.

TaqMan® PreAmp Cells-to-C_T[™] Kit procedure

IMPORTANT! 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 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 begin

- 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.

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 Appendix A, “(Optional) Checking preamplification uniformity” on page 23.

Perform cell lysis

1. **Wash cells in cold PBS in the culture plate, or wash and transfer $\leq 10^5$ 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 Appendix A, “Pilot Experiment” on page 21. Using too many cells per lysis reaction may result in incomplete lysis and/or inhibition of RT-PCR.

For 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 μ L 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.
- c. Wash cells in cold (4°C) PBS by resuspending them in ~0.5 mL PBS per 10⁶ cells (or ~50 µL PBS for ≤10⁵ 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.

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

- d. Resuspend cells in fresh, cold 1X PBS so that 5 µL will contain the desired number of cells for a single lysis reaction (10–10⁵ 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 the table below:

Component	Each 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-C_TTM lysates may appear cloudy at room temperature—this is expected.

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

5. Add 5 µL Stop Solution, or 6 µL Stop Solution with XenoRNATM Control, and mix 5 times.

- a. (Optional) To include an endogenous control using the TaqMan® Cells-to-C_TTM Control Kit, prepare Stop Solution containing XenoRNATM Control for the samples in the experiment at hand. Add 1 µL XenoRNATM Control per 5 µL Stop Solution.
- b. Pipet 5 µL of Stop Solution alone or 6 µL of Stop Solution with XenoRNATM 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! Thoroughly mix the Stop Solution into the lysate.

6. Incubate for 2 min at room temperature.

- a. Incubate for 2 min at room temperature (19–25°C).

Do not allow Cells-to-C_TTM 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.

Perform reverse transcription (RT)

1. Program the thermal cycler for the RT as shown in the table below:

	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 the table below, assemble an RT Master Mix for all the reactions plus ~10% overage in a nuclease-free microcentrifuge tube on ice.

The table below 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-C_TTM 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.

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.

Preamplification

Pre-amplification 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 ≤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 XenorRNA[™] Gene Expression Assays included with the TaqMan® Cells-to-C_T[™] Control Kit.
- Before performing preamplification with limited biological samples, we recommend checking whether all amplicons are amplified without bias using the experiment described in Appendix A, “(Optional) Checking preamplification uniformity” on page 23.

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 20× 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 1× TE buffer so that each assay is at a final concentration of 0.2×. For the above example, add 500 µL 1× 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 the table below.

	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 the table below 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.

Component	Amount
TaqMan® PreAmp Master Mix	25 µL
Pooled assay mix (0.2×)	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.
- b. Upon completion, immediately remove the tubes or plate from the thermal cycler and place it on ice.

IMPORTANT! 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 with 1X TE, based on the number of preamplification cycles, as shown in the table below.

	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 µL

Perform real-time PCR

1. Program the real-time PCR instrument.

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-C_T™ Control Kit are labeled with FAM™ dye and a nonfluorescent quencher.

	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 the PCR Cocktail and aliquot into reaction tubes/plates.

- a. Calculate the number of PCR assays in the experiment. Using the table below, assemble a PCR Cocktail for all the reactions plus ~10% overage in a nuclease-free microcentrifuge tube at room temp. The table below 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.

Component	20 µL PCRs each rxn	50 µL PCRs each rxn
TaqMan® Gene Expression Master Mix (2X)	10 µL	25 µL
TaqMan® Gene Expression Master Mix† (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-C_T™ Kit.

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

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

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 the table below.

Component	20 µL PCRs	50 µL PCRs
PCR Cocktail	15 µL	37.5 µL
Diluted PreAmp product (from step 5 on page 15)	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.

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

Troubleshooting

Observation	Possible cause	Recommended action
No PCR product or unexpected PCR products	Problems with adding or mixing the Stop Solution	<p>Components in the Lysis Solution may inhibit RT-PCR if they are not fully inactivated by the Stop Solution.</p> <ul style="list-style-type: none"> • 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 this 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	<p>Too many cells were used in the lysis reaction</p> <p>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.</p> <ul style="list-style-type: none"> • Generally $\leq 10^5$ cells can be used successfully in the Cells-to-C_T™ 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 (Appendix A). <p>Too much PBS was left on the cells, diluting the Lysis Solution</p> <p>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 10^5 cell.</p>

Observation	Possible cause	Recommended action
No PCR product or unexpected PCR products (continued from page 17)	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 preamplification	<ul style="list-style-type: none"> • 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. <p>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.</p> <ul style="list-style-type: none"> • Preamplification reaction products must be diluted as described in step 5 before using them in PCR.
No PCR product or unexpected PCR products (continued from page 17)	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: <ul style="list-style-type: none"> • Verify that the TaqMan® PreAmp Cells-to-C_T[™] Kit procedure is working by including XenoRNA[™] Control (from the TaqMan® Cells-to-C_T[™] Control Kit, sold separately) in the sample in step 5a on page 11. Then use the XenoRNA[™] TaqMan® Gene Expression Assay to amplify a XenoRNA[™] target following the instructions in section “Perform real-time PCR” on page 15. 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-C_T[™] 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-C_T[™] lysate.



Observation	Possible cause	Recommended action
<p>RT-PCR products in the negative control reactions</p>	<p>PCR products in the no-template PCR control</p>	<p>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.</p> <ul style="list-style-type: none"> 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 DNAZap™ Solution (Cat. no. 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.
<p>RT-PCR products in the negative control reactions (continued from page 19)</p>	<p>PCR products in the minus-RT control</p>	<p>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:</p> <ul style="list-style-type: none"> Be sure to mix DNase I into the Lysis Solution thoroughly in step 2 on page 11 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 3 on page 11) using Lysis Solution that is at room temperature, and make sure that lysis reactions occur at room temperature (19–25°C). <p>If PCR products are still seen in minus-RT control reactions, try the following two suggestions in the order shown:</p> <ul style="list-style-type: none"> Increase the lysis reaction incubation time in step 4 on page 11 to 8 minutes. Use Lysis Solution that has been warmed up to 25°C for cell lysis (step on page 11).





Pilot Experiment

The purpose of this pilot experiment is to identify the maximum number of cells to use in TaqMan® PreAmp Cells-to- C_T ™ Kit 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- C_T ™ Control Kit (Cat. no. 4386995), we recommend adding XenoRNA™ Control to the Stop Solution used to prepare Cells-to- C_T ™ 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- C_T ™ 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- C_T ™ 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

Procedure

1. Harvest, count and wash cells.
Follow the instructions for harvesting cells in step 1 on page 10, but follow the instructions in the next step for resuspending them in PBS.
2. Dilute cells to 2×10^5 cells/ μ L.
Prepare a cell suspension containing 2×10^5 cells/ μ L. Keep the cell suspension on ice.
3. Make 5 serial dilutions of the cells in 5-fold increments
 - a. Prepare 5 tubes containing 45 μ L of cold 1X PBS in ice.
 - b. Transfer 5 μ L of the 2×10^5 cells/ μ L to the first tube (1:5 dilution) and mix gently but thoroughly. Continue making the serial dilutions by transferring 5 μ L of each solution to the subsequent tube to finish with 5 suspensions containing 2×10^4 , 2000, 200, 20, and 2 cells per μ L.
4. Transfer cells to reaction containers with 3 replicates.
Transfer 5 μ L 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 μ L of Lysis Solution and incubate at room temp for 5 min.
 - a. Add 50 μ L of Cells-to- C_T TM 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 μ L Stop Solution, mix, and incubate at room temp for 2 min
 - a. (Optional) Add XenoRNATM Control to the Stop Solution.
Mix 16.5 μ L XenoRNATM Control with 82.5 μ L Stop Solution for use in the next step.
 - b. Add 5 μ L Stop Solution alone or 6 μ L Stop Solution with XenoRNATM 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 “Perform cell lysis” on page 10 to “Perform real-time PCR” on page 15 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- C_T TM Control Kit.
 - If XenoRNATM Control was added to samples, it can be amplified in a separate PCR using the XenoRNATM TaqMan[®] Gene Expression Assay included in the Cells-to- C_T TM 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**

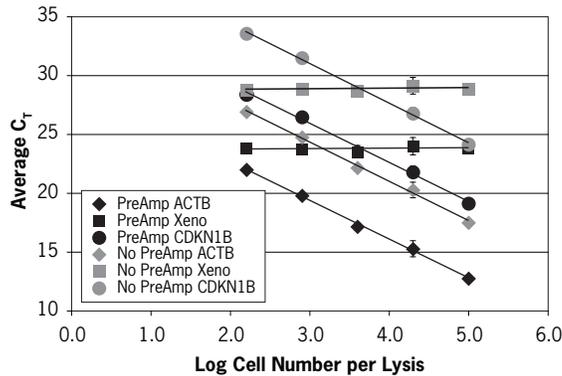
The C_T values from the XenoRNA™ Control should be consistent ($\pm 1 C_T$) regardless of the number of cells in the lysis reaction, indicating that no RT-PCR inhibitors are present in the Cells-to- C_T ™ lysate. If C_T values begin to increase at higher numbers of cells per lysis reaction, it indicates 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 2 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.



(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- C_T ™ lysate, compare normalized C_T values from preamplified Cells-to- C_T ™ lysate to those from lysates that were not preamplified.

1. Prepare Cells-to- C_T ™ lysate.

Follow the instructions in section “Perform cell lysis” on page 10 to prepare 50 μ L of Cells-to- C_T ™ lysate from a non-limited samples, such as a control sample.

2. Reverse transcribe a portion of the lysate.
 Follow the instructions in section “Perform real-time PCR” on page 15 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- C_T^{TM} 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 “Preamplification” on page 13 to preamplify a portion of the cDNA.
 - Include an endogenous uniformity reference gene in your pool. For human gene expression assays, we recommend 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 “Perform real-time PCR” on page 15.
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 (Part no. 4364016)*
 - *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Relative Quantitation Getting Started Guide (Part no. 4347824).*
 - a. Calculate the average C_T values for each assay. [Described in the calculations below as $\text{avg } C_{T(\text{target } X)}$]

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:

$$\Delta C_{T(\text{cDNA})} = \text{avg } C_{T(\text{target } X)} - \text{avg } C_{T(\text{Uniformity of gene})}$$
 The purpose is to normalize each individual target to the desired uniformity reference gene when using cDNA that is not preamplified. The $\Delta C_{T(\text{cDNA})}$ 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:

$$\Delta C_{T(\text{Preamp})} = \text{avg } C_{T(\text{target } X)} - \text{avg } C_{T(\text{Uniformity of gene})}$$
 The purpose is to normalize each individual target to the desired uniformity reference gene when using preamplified cDNA. The $\Delta C_{T(\text{cDNA})}$ represents the normalized C_T for the preamplified cDNA.

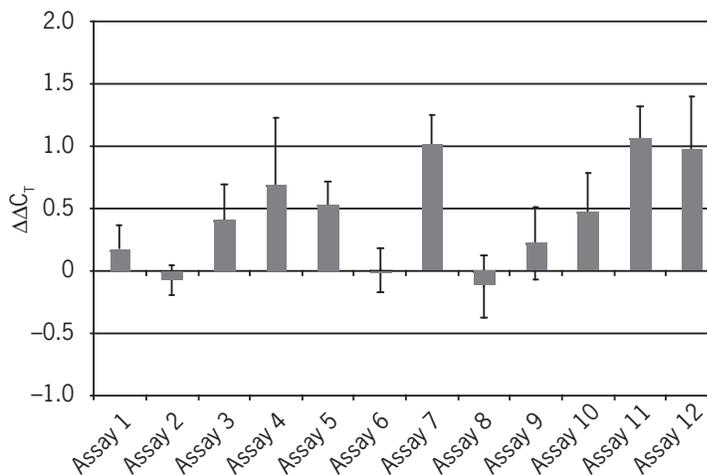
- d. Calculate the $\Delta\Delta C_T$ for each assay between cDNA and preamplified cDNA by subtracting the ΔC_T value for cDNA (step b) from the ΔC_T for multiplex preamplification (step c):

$$\Delta\Delta C_T = C_{T(\text{Preamp})} - \Delta C_{T(\text{cDNA})}$$

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 within 1.5 C_T values of zero. The following figure shows an example of preamplification uniformity results for 12 assays.

Figure 3 Example of a preamplification uniformity experiment

Note that all 12 assays evaluated showed uniform preamplification ($\Delta\Delta C_T$ within ± 1.5)





Appendix A Pilot Experiment

(Optional) Checking preamplification uniformity

B

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-C_T[™] Kits were developed using Applied Biosystems® 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-C_T[™] Kit 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
- Phosphate-buffered saline (PBS), for example diluted from Life Technologies 10X PBS, (Part nos. AM9624 and AM9625)
- Molecular biology grade TE buffer: 10 mM Tris-HCl (pH ~8), 1 mM EDTA, for example Life Technologies TE, pH 8.0 (Cat. no. AM9849)
- TaqMan® Gene Expression Assay for real-time PCR of the target(s) of interest (Cat. nos. 4331182, 4351372).
- (optional) TaqMan® Cells-to-C_T[™] Control Kit (Cat. no. 4386995)

Related products available from Life Technologies

<p>TaqMan® Cells-to-C_T™ Control Kit Cat. no. 4386995</p>	<p>Designed for use with TaqMan® Cells-to-C_T™ 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.</p>
<p>TaqMan® Gene Expression Assays Cat. nos. 4331182 and 4351372</p>	<p>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, <i>Arabidopsis</i>, <i>Drosophila</i>, <i>C. elegans</i>, <i>C. familiaris</i> (dog), and Rhesus macaque genes, and Custom TaqMan® Gene Expression Assays are also available.</p>
<p>TaqMan® MicroRNA Assays</p>	<p>TaqMan® MicroRNA Assays quantitate miRNAs with the specificity and sensitivity of TaqMan® assay chemistry. Use a simple, two-step strategy of reverse transcription with an miRNA-specific primer, followed by real-time PCR with TaqMan® probes. The assays target only mature miRNAs, not their precursors, ensuring biologically relevant results.</p>
<p>Multiplex RT for TaqMan® MicroRNA Assays Cat. no. 4384791 and others</p>	<p>Multiplex RT for TaqMan® MicroRNA Assays are a set of eight predefined reverse transcription primer pools that streamline the process of creating cDNA for TaqMan® assay analysis of 365 unique human miRNAs. Each individual Multiplex RT pool contains up to 48 primers per pool, including two endogenous controls for data normalization. Multiplex RT pools are formulated for use with individual TaqMan® MicroRNA Assays providing better sensitivity and specificity, reduced sample consumption, and quicker miRNA profiling results compared to microarray-based methods.</p>
<p>Applied Biosystems® Real-Time PCR Instruments</p>	<p>Since pioneering real-time PCR, Life Technology has continued to develop the technology to provide more powerful solutions for labs of all sizes. The Life technologies 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.</p>
<p>RNaseZap® Solution Cat. nos. AM9780, AM9782, AM9784</p>	<p>RNaseZap® 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 RNaseZap® Solution.</p>
<p>RNase-free Tubes & Tips</p>	<p>Ambion® RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free.</p>
<p>RT-PCR Grade Water Cat. no. AM9935</p>	<p>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.</p>

<p>Plastic Consumables for PCR</p>	<p>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.</p>
<p>DNAZap™ Solution Cat. no. AM9890</p>	<p>DNAZap™ 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.</p>

B

Appendix B Materials Not Provided with the Kit
Related products available from Life Technologies



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.
-

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.
-

Documentation and Support

Obtaining SDSs

Safety Data Sheets (SDSs) are available from www.lifetechnologies.com/sds

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

Obtaining support

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

www.lifetechnologies.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

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at www.lifetechnologies.com/termsandconditions. If you have any questions, please contact Life Technologies at www.lifetechnologies.com/support.



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