Cells-to-Signal™ Kit

Reverse Transcription without RNA Isolation

Part Numbers AM1724, AM1726

Cells-to-Signal™ Kit

(Part Number AM1724, AM1726)

Protocol

P/N 1724M Revision C **Revision Date: September 30, 2010**

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I. Introduction

A. Product Description and Background

RT-PCR is one of the most common methods used for measuring mRNA levels in biological samples. Traditionally, RNA has been isolated from the sample to remove RNases and reverse transcriptase inhibitors before being used as substrate for RT-PCR. RNA isolation is fairly time-consuming, and with small samples, the isolation can lead to loss of RNA. The Cells-to-Signal™ technology (patent pending) produces cDNA from cultured mammalian cells without prior RNA isolation. By eliminating the RNA isolation step, RT-PCR analysis of a large number of samples becomes much simpler and faster.

The Cells-to-Signal Kit is based on Ambion Cells-to-cDNA™ technology, but further simplifies the procedure by eliminating high temperature incubation and DNA digestion, making the procedure even more user-friendly and easy to automate.

A control RNA is included in the kit for optimization and normalization. The Control RNA is a 1036 nt random sequence RNA with no homology to known genomic sequences. It includes a poly(A) tail so that it can be reverse transcribed with the Oligo(dT) Primers like a natural mRNA.

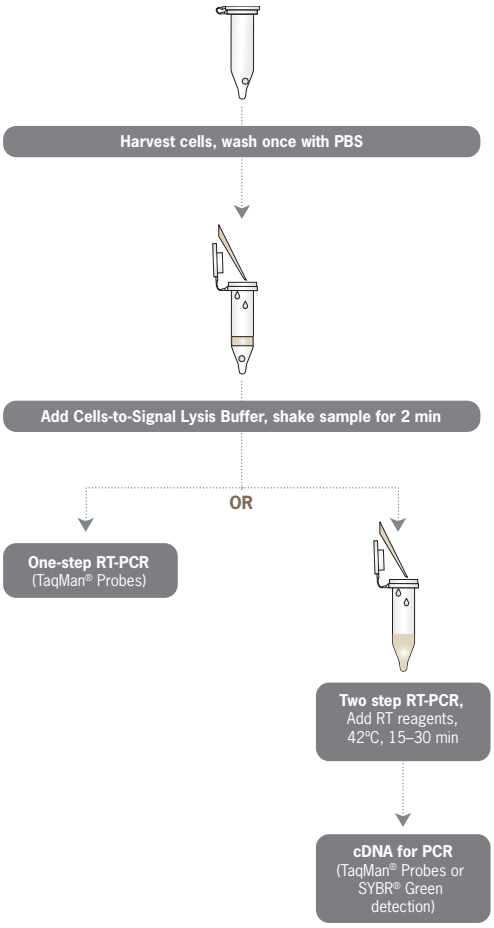
Procedure overview

The Cells-to-Signal technology uses a single buffer for cell lysis and RNase inactivation. The lysate can then be added directly to a reverse transcription (RT) reaction. Generally lysates can be prepared for RT-PCR in less than 10 minutes.

The procedure is shown in Figure 1. Cultured cells are washed with PBS and then lysed in the Cells-to-Signal Lysis Buffer by shaking/vortexing for 1–2 min at room temperature. This treatment has two important effects: it ruptures the cells, releasing the RNA into the Lysis Buffer; and it inactivates endogenous RNases, protecting the RNA from degradation. Once lysis is complete, the cell lysate is ready for reverse transcription and PCR using either a one-step or two-step RT-PCR strategy. The whole process from lysing cells to assembling the one step RT-PCRs can be fully automated. Visit the automation resource page for recommendations at:

www.ambion.com/techlib/resources/automation/

Figure 1. Cells-to-Signal Procedure Overview



Applications

The Cells-to-Signal Kit can be used in any application where RT-PCR or real-time RT-PCR* is used to analyze mRNA from cultured cells. The Cells-to-Signal Kit is well suited for high throughput mRNA quantitation in RNAi studies, or real-time analysis of large numbers of differentially treated cell cultures. For example, the regulation of an mRNA can be followed as cells are treated with increasing concentrations of a

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particular chemical (Sumida 1999). The Cells-to-Signal process is also useful for screening compounds for their effects on mRNA expression, or for performing time course experiments (Su 1997).

The kit is designed for use with TaqMan® probes for both one-step and two-step real-time qRT-PCR, or SYBR Green with two step qRT-PCR. A linear response can be achieved with up to 1000 cells/µL Lysis Buffer as shown in Figure 2 (linear range varies with the cell type and mRNA of interest). Figure 3 illustrates the sensitivity of the Cells-to-Signal technology.

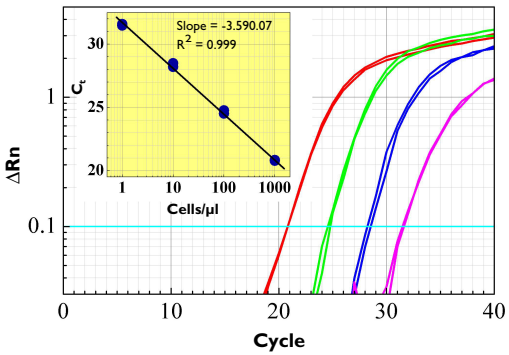

Figure 2. Real-time RT-PCR Using the Cells-to-Signal™ Kit

A dilution series of HeLa cells was processed with the Cells-to-Signal Kit according to this protocol. The lysate was used in one-step real-time RT-PCR using SuperTaq™ and TaqMan® Gene Expression Assays (Applied Biosystems) primer/probe sets to PRKCL1. Results were plotted on a semilog graph to show linear recovery of mRNA over a cell input range of 1–10^3 cells/µL Cell Lysis Buffer.

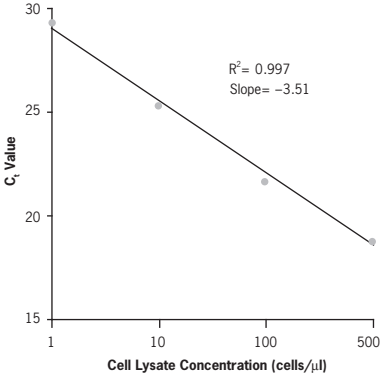

Figure 3. Linear detection of VEGF using the Cells-to-Signal™ Kit

K562 cells were serially diluted and cell lysates were prepared using the Cells-to-Signal Kit to give the indicated lysate concentrations. 2 µL of each cell lysate was used as a template for amplification of VEGF in a 10 µL one-step real-time RT-PCR in a 384 well plate using an ABI 7900-HT real-time thermal cycler.

Cell types compatible with the Cells-to-Signal Kit

The cell types listed below have been shown to work well with the Cells-to-Signal Kit:

- BJ: Human non-transformed foreskin cells
- HeLa: Human negroid cervix epitheloid carcinoma
- HeLa S3: Human negroid cervix epitheloid carcinoma
- K562: Human caucasian, pleural effusion, leukemia, chronic myeloid
- MCF-7: Human caucasian, breast, adenocarcinoma
- NHDF-neo, Human, primary cell line
- SKNAS: Human, bone marrow, neuroblastoma

B. Materials Provided with the Kit and Storage Conditions

The Cells-to-Signal Kit contains reagents and controls for the production of cDNA directly from cell lysates, for use in PCR. Alternatively, the cell lysate produced with the kit can be used in one step RT-PCR. The Cells-to-Signal Kit does ***not*** include thermostable DNA polymerase.

Storage at –20°C should be in a non frost-free freezer.

P/N AM1724 30 rxn	P/N AM1726 100 rxn	Component	Storage
20 mL	2 x 20 mL	1X PBS Buffer pH 7.4	any temp*
3 mL	11 mL	Cells-to-Signal Lysis Buffer	any temp*
1.75 mL	2 x 1.75 mL	Nuclease-free Water	any temp*
15 µL	55 µL	50X ROX Standard	–20°C
75 µL	275 µL	10X RT Buffer	–20°C
30 µL	110 µL	M-MLV RT (25 U/µL in storage buffer)	–20°C
30 µL	110 µL	RNase Inhibitor (10 U/µL in storage buffer)	–20°C
120 µL	440 µL	dNTP Mix (2.5 mM each dNTP)	–20°C
60 µL	220 µL	Random Decamers (50 µM in TE buffer)	–20°C
60 µL	220 µL	Oligo(dT) Primers (50 µM in TE buffer)	–20°C
10 µL	10 µL	Control RNA (1 ng/µL)	–20°C
30 µL	110 µL	Control RNA Primers (10 µM each)	–20°C
1 mL	1 mL	RNA Storage Solution	–20°C

* Store these components at –20°C, 4°C, or room temp.

C. Materials Not Provided with the Kit

- Compatible thermostable DNA polymerase

Conventional thermostable DNA polymerases	Hot start thermostable DNA polymerases
Ambion SuperTaq™	Sigma JumpStart Taq
ABI AmpliTaq®	Invitrogen Platinum® Taq
Roche Taq	Eppendorf HotMaster® Taq

- 10X PCR Buffer (for two-step RT-PCR)
  The 10X RT Buffer supplied with this kit is optimized for both reverse transcription, and one-step RT-PCR. For two-step RT-PCR, the user must supply 10X PCR buffer and $MgCl_2$.
- PCR primers/probe for the target of interest
  The Cells-to-Signal Kit is compatible with Applied Biosystems' TaqMan Gene Expression Assays.

D. Related Products Available from Applied Biosystems0

*SuperTaq™ Polymerase P/N AM2050, AM2052	Thermostable DNA Polymerase (includes 10X buffers and dNTPs)
†SuperTaq™ Plus P/N AM2054, (50U) P/N AM2056, (250U)	Extended Range Thermostable DNA Polymerase Super Taq Plus has a proof reading activity, and produces significantly higher yields of PCR products than ordinary Taq polymerase (includes 10X buffers and dNTPs)
DNA*Zap*™ Solution P/N AM9890	DNA degradation solution to avoid PCR contamination. This mixture is able to degrade high levels of contaminating DNA and RNA from surfaces instantly.
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 our web or print catalog	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.
QuantumRNA™ 18S Internal Standards P/N AM1716–AM1718	Exclusive kit containing blocked 18S primers that can be used in conjunction with the supplied unblocked 18S primers to attenuate the amount of PCR product amplified from 18S RNA. Use this kit to do multiplex relative RT-PCR, comparing the RT-PCR signal from the gene of interest to that amplified from the desired fraction of 18S RNA.
Cells-to-cDNA™ II Kit P/N AM1722, AM1723	The Cells-to-cDNA II Kit (patent pending) is designed for reverse transcription directly from mammalian cell lysate, without RNA isolation. It is ideal for synthesizing cDNA from small numbers of cells, numerous cell samples, or for labs that are not equipped for RNA isolation.

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II. Planning the Experiment and Cell Harvesting

A. Primer and Probe Selection for Real-time PCR

There are many programs available for primer design, such as Primer Express® (Applied Biosystems), as well as many free web-based programs. If possible, primers should be designed to avoid regions of high secondary structure (free energies less than –14 kcal/mol), as these may inhibit read-through of reverse transcriptase (Pallansch 1990). The amplicon size should be ~100 bp, and sequence-specific TaqMan probes should be used when the highest specificity is desired.

To avoid amplification of genomic DNA, one of two approaches is suggested for primer design (Figure 4):

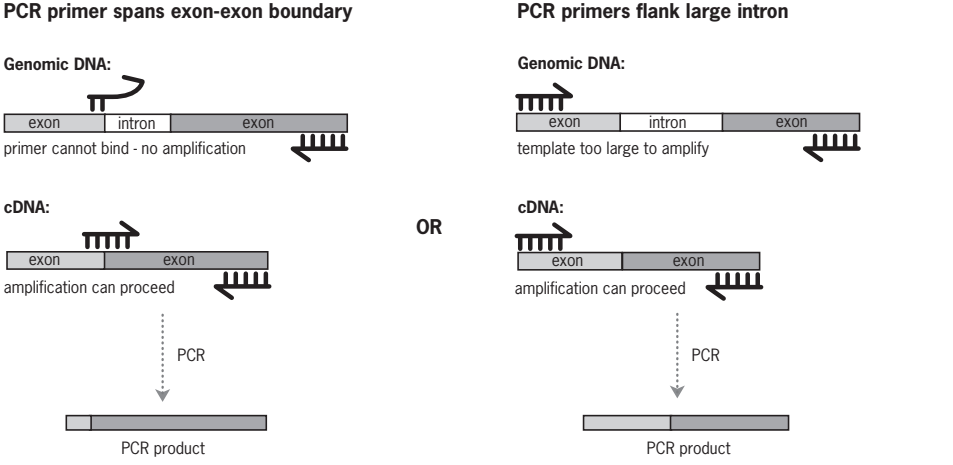
Span an exon-exon boundary

Choose primers that span an exon-exon boundary in the target mRNA so that one-half of the primer hybridizes to the 3' end of one exon and the other half to the 5' end of the next exon. Primers designed this way will not anneal to genomic DNA, but will anneal to cDNA synthesized from spliced mRNAs. We recommend Applied Biosystems' Gene Expression Assays, many of which are designed to span exon-exon junctions.

Flank a large intron

Choose primers on either side of a large intron so that, under the cycling conditions used, no amplification will occur from the genomic DNA template.

Figure 4. PCR Primer Designs to Minimize Genomic DNA Amplification.



B. Optimizing the RT-PCR

One-step or two-step RT-PCR?

We provide procedures for both one-step and two-step RT-PCR. However, we recommend using the one-step RT-PCR procedure with the Cells-to-Signal Kit. The major advantage of one-step RT-PCR is that it requires fewer manipulations, and therefore yields more consistent results in less time.

Optimize the PCR with purified RNA first

Optimize the PCR (e.g. denaturation temperature, cycle times, and primer sequence) with purified RNA as template for reverse transcription before trying to amplify cDNA prepared with the Cells-to-Signal Kit.

Controls

We suggest including two negative controls with every reaction:

- A ***minus-template control*** containing all the reaction components except the cell lysate (substitute water): This control verifies that none of the PCR reagents are contaminated with DNA.
- A ***minus-RT control*** containing all the reaction components except the M-MLV RT (substitute water): This control demonstrates that the template for the PCR product was cDNA, and not genomic DNA.

Select a thermostable DNA polymerase

The Cells-to-Signal Kit contains reagents for reverse transcription, but thermostable DNA polymerase (available from Ambion) and gene specific primers and probe, for real-time, must be supplied by the user. See section *I.C. Materials Not Provided with the Kit* on page 5 for a list of thermostable DNA polymerases that are compatible with this procedure.

C. Cell Harvesting Procedures

Choosing the appropriate number of cells

The maximum number of cells that can be effectively lysed in 100 µL of Lysis Buffer varies somewhat according to cell type, but is generally $\leq 5 \times 10^4$ cells. Higher cell concentrations may result in inhibition of RT-PCR. We recommend that you conduct a pilot experiment if you intend to use more than 5×10^4 cells/100 µL reaction (see section *III.D. Pilot Experiment* on page 16 for instructions).

Is the PBS wash required?

Normally cells should be washed in cold PBS to remove the serum in the medium, which contains ribonucleases and inhibitors of reverse transcription. It may be possible to omit the wash step if the growth medium has very little serum or if only a few cells will be added to the Lysis Buffer. (This can be determined using the Control RNA, see Section *IV.A. Positive Control Reactions* on page 18.)

Use the procedure appropriate for the culture vessel and the adherence properties of the cells

The type of vessel in which the cells were grown and the adherence properties of the cells will dictate the method used for harvesting. Use the method commonly employed in your lab to harvest your specific cells, or follow the instructions provided below for adherent cells grown in a 96-well plate (1), adherent cells grown in other vessels (2), or cells in suspension (3).

1. Adherent cells grown in a 96 well plate

a. Count or estimate the number of cells per well. Make sure that each well contains fewer than 5×10^4 cells.
For $>5 \times 10^4$ cells per well, we recommend testing for inhibition of RT-PCR by conducting the pilot experiment described in section III.D on page 16.

b. Aspirate the culture medium from the wells, and add 200 µL of cold 1X PBS to each well.

c. Aspirate the PBS from the well. There should be no more than 20 µL of residual PBS.
Proceed to cell lysis (step III.A.3** on page 11**).

2. Adherent cells grown in other vessels

If adherent cells are grown in any vessel larger than a 96-well plate, they must be detached from the growing surface. Any method can be used to detach the cells as long as they remain intact through the procedure.

a. Detach cells and aliquot up to 5×10^4 cells into microfuge tubes or wells of a 96-well U-bottom plate (use the method routinely employed in your laboratory for the particular cell type).

IMPORTANT
If trypsin is used to detach cells, inactivate it before proceeding.

b. Pellet the cells at 4°C, discard the growth medium (e.g. by aspiration), and place the cells on ice.
Most cell types can be effectively pelleted by centrifugation at 1,200 x g for 5 min (use the method routinely employed in your laboratory for the particular cell type).

c. Wash cells at least once with cold 1X PBS (4°C) by adding 200 µL 1X PBS and agitating gently to resuspend the cell pellets.

d. Repellet the cells and remove the 1X PBS by aspiration. There should be no more than 20 µL of residual PBS.
Proceed to cell lysis (step III.A.3** on page 11**).

3. Suspension cells

a. Count or estimate the cell concentration, and transfer $\leq 5 \times 10^4$ cells into microfuge tubes or wells of a 96 well U-bottom plate.

b. Pellet the cells at 4°C, discard the growth medium (e.g. by aspiration), and place the cells on ice.

Most cell types can be effectively pelleted by centrifugation at 1,200 x g for 5 min (use the method routinely employed in your laboratory for the particular cell type).

c. Wash cells at least once with 200 µL cold (4°C) 1X PBS. Agitate gently to resuspend the cell pellets.

d. Repellet the cells, and remove the 1X PBS by aspiration. There should be no more than 20 µL of residual PBS.
Proceed to cell lysis (step III.A.3 on page 11).

III. Cells-to-Signal Procedure

A. Cell Lysis

1. Bring reagents to temp

You will need the following reagents at the indicated temperatures for harvesting and lysing cells:

Component	Temperature
1X PBS Buffer	4°C
Cells-to-Signal Lysis Buffer	room temp

2. Harvest cells and wash in cold PBS

Harvest up to 5 x 10^4 cells per reaction according to the established procedures of your lab then wash cells in 200 µL cold PBS. See section II.C starting on page 8 for suggestions on harvesting and washing cells.

3. Add 100 µL Cell-to-Signal Lysis Buffer

a. Add 100 µL Cell-to-Signal Lysis Buffer (***warmed to room temp***) to each cell sample.

b. (**Optional**) Add Control RNA.
See section *IV.A. Positive Control Reactions* on page 18 for a general discussion on using the Control RNA.

   i. Dilute the Control RNA 1:100 in RNA Storage Solution to 10 pg/µL.

   ii. Add 2 µL of the 10 pg/µL diluted Control RNA into 100 µL of Cell Lysate (if control RNA will be used for every sample, premix diluted Control RNA with Cells-to-Signal Lysis Buffer).

4. Shake gently for 2 min

Shake the sample gently for 2 min to lyse cells.

5. Analyze or store samples

Proceed to RT-PCR analysis, or store the lysate for later analysis. Cells-to-Signal lysate can be left at room temp if it will be processed within 4 hr. Store the lysate at –20°C or –80°C for up to 2 months.

B. RT-PCR Instructions

Cells-to-Signal lysates can be used for either one step or two step RT-PCR. Follow the instructions in section C.I below for one step RT-PCR, or in section C.II for two step RT-PCR.

C.I. One-step RT-PCR

1. Assemble the reaction

Assemble the following on ice in thin-walled microfuge tubes.

NOTE

Prepare an RT-PCR master mix to maximize uniformity and minimize labor when the experiment includes multiple samples.

Amount	Component
≤5 µL	Cell lysate (not to exceed 20% of the final volume)
2.5 µL	10X RT Buffer
4 µL	dNTP Mix (2.5 mM each)
0.5 µL	50X ROX Standard*
1 µL	RNase Inhibitor
1 µL	M-MLV Reverse Transcriptase
1 µL	PCR primers (mixture of 10 µM each primer)
1 µL	TaqMan Probe (2 µM)†
1 unit	Thermostable DNA polymerase (0.2 µL of 5U/µL)
to 25 µL	Nuclease-free Water (8.8 µL in this example)

* With Stratagene's Mx4000® qPCR system, use 1/10 the amount of ROX Standard shown in the table above, i.e., use the ROX Standard at a final concentration of 0.1 X.

† This reagent is necessary for real-time PCR using TaqMan detection; leave it out for end-point PCR. For real-time PCR using detection methods other than TaqMan, modify the reaction as needed.

2. Mix the reaction

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

3. Incubate the RT-PCR

Real-time RT-PCR

Cycle as follows for one-step real-time RT-PCR:

	Stage	Reps	Temp	Time
Reverse transcription	1	1	42°C	15 min
RT inactivation/ initial denaturation	2	1	95°C	10 min
Amplification	3	40	95°C 60°C	15 sec 1 min

Endpoint PCR

For endpoint PCR experiments (not real-time), where amplicon sizes are usually longer than 100 bp, we recommend carrying out three-stage PCR with the annealing temperature suggested by your primer design

software. The optimal temperature and cycling times for your primer and template combination may have to be determined empirically. Here is a typical example of cycling conditions:

	Stage	Reps	Temp	Time
Reverse transcription	1	1	42°C	15 min
RT inactivation/ initial denaturation	2	1	94°C	2 min
Amplification	3	35	94°C	30 sec
			55°C	30 sec
			75°C	2 min
Final extension	4	1	75°C	5 min

C.II. Two-step RT-PCR

Primers for reverse transcription

Type of primers

Reverse transcription reactions in two-step RT-PCR are primed with one of the following types of primers:

- random primers: random sequence oligonucleotides
- oligo(dT) primers: oligo$(dT)_{17–24}$ oligonucleotides
- gene specific primers: oligonucleotide that can hybridize with the specific RNA under study

Past experience with a particular target may indicate which type of primer is preferable, either for maximizing yield or for maximizing specificity of the RT-PCR product. In the absence of prior experience, it may be desirable to compare the yield and purity of products between reactions primed with random decamers, oligo(dT), and the reverse PCR primer; Ambion scientists observed better sensitivity using oligo(dT) primers than random decamers.

Concentration of primers for RT

We find that a final concentration of 5 µM of the Random Decamers or the Oligo(dT) Primers is optimal in most reactions. If a gene specific primer is used to prime the RT, its final concentration should be 0.25–5 µM (Innis 1990).

1. Assemble the reverse transcription reaction

Assemble the following in a nuclease-free microfuge tube on ice.

NOTE

Prepare an RT master mix to maximize uniformity and minimize labor when the experiment includes multiple samples.

Amount	Component
≤4 µL	Cell lysate (not to exceed 20% of final volume)
4 µL	dNTP Mix
2 µL	First-strand primers*
2 µL	10X RT Buffer
1 µL	M-MLV RT (for the minus-RT control add water instead of M-MLV RT)
1 µL	RNase Inhibitor
to 20 µL	Nuclease-free Water (6 µL in this example)

* Use 2 µL of the supplied Random Decamers or Oligo(dT) Primers. If you use a gene specific primer, its final concentration should be 0.25–5 µM.

2. Mix the reaction

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

3. Incubate at 42°C for 15–30 min

Incubate at 42°C for 15–30 min for reverse transcription.

4. Inactivate the reverse transcriptase at 92–95°C for 10 min

Incubate the reaction mix at 92–95°C for 10 min to inactivate the reverse transcriptase.

Store the reaction at –20°C or proceed to the PCR amplification.

5. Assemble the PCR

Assemble the components on ice in thin-walled microfuge tubes.

Prepare a PCR master mix to maximize uniformity and minimize labor when the experiment includes multiple samples.

NOTE

Ambion recommends a hot start for PCR reactions. At a minimum, assemble reactions on ice, start preheating the thermal cycler to 95°C, and put the tubes in the thermal cycler when it has attained ≥85°C.

Amount	Component
5 µL	cDNA from RT reaction (for the minus-template control use water instead of cDNA)
2.5 µL	10X real-time PCR Buffer, –$MgCl_2$
2 µL	dNTP Mix (2.5 mM each)
5 µL	25 mM $MgCl_2$ *
0.5 µL	50X ROX Standard
1 µL	TaqMan Probe (2 µM)†
1 µL	PCR primers (mixture of 10 µM each primer)
1 unit	Thermostable DNA polymerase (0.2 µL of 5 U/µL)
to 25 µL	Nuclease-free Water (7.8 µL in this example)

* We recommend a final $MgCl_2$ concentration of 5 mM in the PCRs.

† Include ROX Standard for real-time PCR using TaqMan detection; for end-point PCR, replace it with Nuclease-free Water. For real-time PCR using detection methods other than TaqMan, modify the reaction as needed.

6. Mix the reaction

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

7. Incubate the PCR

Real-time PCR cycling conditions

	Stage	Reps	Temp	Time
Initial denaturation	1	1	95°C	5 min
Amplification	2	40	95°C	15 sec
			60°C	1 min

NOTE

Hot-start DNA polymerases may require longer heating at 95°C; follow manufacturer's recommendations for enzyme activation.

Endpoint PCR cycling conditions

For endpoint PCR experiments (not real-time), where amplicon sizes are usually longer than 100 bp, we recommend carrying out three-stage PCR with the annealing temperature suggested by your primer design software. The optimal temperature and cycling times for your primer and template combination may have to be determined empirically. Here is a typical example of cycling conditions:

	Stage	Reps	Temp	Time
Initial denaturation	1	1	94°C	2 min
Amplification	2	35	94°C	30 sec
			55°C	30 sec
			75°C	2 min
Final extension	3	1	75°C	5 min

D. Pilot Experiment

RT-PCR is inhibited if too many cells are used; the optimal number of cells per reaction varies among different cell types. To determine the maximum number of cells of a particular type that can be used in the procedure without causing inhibition of RT-PCR, conduct the pilot experiment described here. In this simple experiment, a serial dilution of the cells is made, and different numbers of cells are lysed in Lysis Buffer containing the Control RNA. Then the lysates are subjected real-time RT-PCR, and the signal from the Control RNA in the experimental samples is compared to the signal from a control sample without cells to ascertain the maximum numbers of cells that can be used in a reaction with out inhibiting reverse transcription of the control.

1. Harvest and count cells

Follow the instructions for harvesting cells in section *II.C. Cell Harvesting Procedures* on page 8.

Note that some adherent cell types can be collected by simply scraping them up with a rubber spatula (Freshney, 1987), but this method is not recommended for pilot experiments because cells may not be completely dispersed in PBS, compromising the accuracy of cell concentrations in serial dilutions.

2. Prepare 20 µL of 1X PBS containing 2 x 10^5 cells

Prepare 20 µL of a cell suspension containing 10^4 cells/µL. This will serve as the most concentrated sample.

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

a. Prepare 4 tubes containing 16 µL of cold 1X PBS in ice.

b. Transfer 4 µL of the 10^4 cells/µL to the first tube (1:5 dilution) and mix gently but thoroughly. Continue making the serial dilutions by transferring 4 µL of each solution to the subsequent tube to finish with 5 solutions containing 10^4, 2 x 10^3, 400, 80, and 16 cells per µL.

4. Lyse 10 µL of cells in 90 µL of Lysis Buffer containing 0.2 pg/µL of Control RNA

a. Prepare 550 µL of Cells-to-Signal Lysis Buffer containing 0.2 pg/µL Control RNA.

i. Dilute 1 µL of the Control RNA into 99 µL RNA Storage Solution for a final concentration of 10 pg/µL, and mix thoroughly.

ii. Add 12 µL of the 10 pg/µL diluted Control RNA to 538 µL Cells-to-Signal Lysis Buffer.

b. Add 10 µL of each cell dilution (from step 3) to 90 µL of Cells-to-Signal Lysis Buffer, and shake gently for 2 min.

c. Add 10 µL of PBS to 90 µL of Cells-to-Signal Lysis Buffer for the no-cells control.

5. Amplify the Control RNA by one or two step RT-PCR

Reverse transcribe and PCR amplify all samples using either one step or two step RT-PCR as described in section *III. Cells-to-Signal Procedure* starting on page 11.

- Use the Control RNA Primers as the PCR primers.
- For TaqMan detection of PCR products, synthesize a TaqMan probe the sequence described in section IV.A *TaqMan probe for the Control RNA* on page 18.
- For two-step RT-PCR use Oligo(dT) Primers to prime the reverse transcription reaction, and incubate the reverse transcription for 30 min.
- The Control RNA Primers should amplify an 83 bp PCR product from the Control RNA.

6. Evaluate results

The highest cell concentration that produces a Control RNA PCR product equivalent to the amount amplified from the no-cells control is considered the maximal cell concentration.

IV. Troubleshooting

A. Positive Control Reactions

Description of the Control RNA and Control RNA Primers

The Cells-to-Signal Kit includes a Control RNA and corresponding Control RNA Primers for use in PCR. The Control RNA can be used to monitor both the efficiency of the RNase inactivation in the lysis step, and any inhibitory effects of the cell lysate on reverse transcription. It can also be used for general troubleshooting.

Control RNA description

The Control RNA is a 1036 nt random sequence RNA with no homology to known genomic sequences. It has a poly(A) tail so that it can be reverse transcribed with the Oligo(dT) Primers.

Instructions for using the Control RNA

The Control RNA is supplied at 1 ng/µL and should be used at a final concentration of 0.2 pg/µL (~0.35 x 10^6 copies/µL). For accurate dilution of the Control RNA, prepare an initial 1:100 dilution in RNA Storage Solution to make a 10 pg/µL solution. Diluted Control RNA should be used in the same day, otherwise the concentration may change due to the adsorption of RNA to the tube wall. Add the diluted Control RNA to each cell lysate (2 µL per 100 µL cell lysate), or add it to the Lysis Buffer before adding cells.

Amplification of the Control RNA

Use the Control RNA Primers in one step or two step RT-PCR following the instructions in section *III. Cells-to-Signal Procedure* starting on page 11. The Control RNA Primers are supplied as a mixture containing 10 µM of each primer. For two step RT-PCR, incubate the reverse transcription reaction for 30 min, and conduct the PCR with 55°C for the annealing temperature.

The Control RNA PCR product is 83 bp

The Control RNA should generate a 83 bp PCR product when amplified with the Control RNA Primers.

TaqMan probe for the Control RNA

For TaqMan detection of real-time PCR amplification of the Control RNA, synthesize a TaqMan probe with the following sequence:

5'-CAAGCGTAAATGCAGCGTCCA-3'

Routine use of the Control RNA

The Control RNA may be mixed with the Cells-to-Signal Lysis Buffer at a final concentration of 0.2 pg/µL, so that every sample contains Control RNA for troubleshooting and normalization.

Troubleshooting with the Control RNA

If no PCR product is generated using the experimental PCR primers, then add 0.2 pg/µL Control RNA to an aliquot of your cell lysate, and attempt to amplify the Control RNA with the Control RNA Primers. Follow the instructions for one-step RT-PCR in section III.C.I on page 12. Be sure to include minus-template and minus-RT controls in the experiment.

Analysis of positive control experiments

Real-time PCR of the Control RNA

Using the standard Procedure described above and in section III, ***the Control RNA will be detected at approximately 20 Ct using TaqMan methodology***.

With real-time RT-PCR, observing good amplification signal using the Control RNA indicates that the RT-PCR is functioning properly. If there is no amplification signal, then there may be a problem with one or more of the RT or PCR components.

Endpoint RT-PCR of the Control RNA

To analyze endpoint RT-PCR products from the positive control reaction, fractionate 10–20 µL of the PCR on a 2% agarose gel stained with ethidium bromide or another nucleic acid stain.

The Control RNA should generate an 83 bp PCR product.

If the 83 bp control PCR product is amplified, it indicates that the RT-PCR worked properly. If the 83 bp Control RNA PCR product is not seen, then there may be a problem with one or more of the RT or PCR components.

B. No PCR Product or Unexpected PCR Products

The PCR requires optimization

Too few PCR cycles

It may be possible to increase the sensitivity of the PCR by increasing the number of cycles performed. In some labs, it is routine to use as many as 40 cycles. However, always run a minus-template negative control to be sure that DNA contaminants are not amplified.

Primer annealing temperature is not optimal

Sometimes unexpected products in an RT-PCR come from non-specific priming of unrelated cDNA sequences during the PCR. Raising the stringency of the PCR by increasing the annealing temperature can often improve results. Try several annealing temperatures to identify the one that works the best.

RNA was degraded prior to adding Lysis Buffer

To avoid RNA degradation, keep cells in PBS on ice at all times before the cell lysis procedure. Take cells off ice just prior to adding Cells-to-Signal Lysis Buffer. Make sure that the Lysis Buffer is at room temp.

RNase in the sample was not completely inactivated

If too many cells per sample are used in the procedure, the RNase in the sample may not be totally inactivated and/or high concentrations of cellular components (debris) may inhibit reverse transcription. Generally 5×10^4 cells or fewer can successfully be used in the Cells-to-Signal procedure, but if PCR fails, try fewer cells. It is also useful to compare the cell number used in the failed experiment to 5–10 fold fewer cells using the Control RNA as an indicator of RT-PCR efficiency. Consider performing a pilot experiment to determine the best concentration of cells for your particular cells type (see section III.D on page 16).

If too much PBS was remaining in the sample when the Cells-to-Signal Lysis Buffer was added, cellular RNases may not be fully inactivated due to dilution of the Lysis Buffer. To avoid diluting the Lysis Buffer, remove as much PBS as possible before adding Lysis Buffer to the cells.

Too much cell lysate was used for RT-PCR

Endogenous inhibitors will reduce the yield of reverse transcription products if too much cell lysate was used in the reverse transcription reaction in step III.B.1 on page 12 of the one-step RT-PCR procedure, or step III.B.1 on page 14 of the two-step RT-PCR procedure. The cell lysate should make up no more than 20% of the final reaction volume.

The sample does not contain the mRNA

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

a. Check that the PCR for the gene of interest works with your PCR primers, reagents, and equipment by using 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-Signal from cells.

b. Verify that the Cells-to-Signal procedure is working by including Control RNA in the sample as described in section IV.A. on page 18. If you are able to amplify the Control RNA, then it is possible that the RNA of interest is not expressed in this tissue culture cells and/or is undetectable with this procedure.

There are unexpected targets in the cDNA

Unexpected PCR products in RT-PCR may represent alternatively spliced forms of a transcript or amplification of a fragment from a related message. Cloning and sequencing of the PCR products can resolve this question. Alternatively, design primers to a unique region of the transcript (e.g. the 3' untranslated region), and try these primers in the PCR.

C. RT-PCR Products in the Negative Control Reactions

Cells-to-Signal was developed mainly for fast, one-step real-time RT-PCR from tissue culture cells. There is no DNase treatment involved in this process. Ambion highly recommends using primers that span an exon-exon boundary or flank large introns to eliminate the amplification of genomic DNA. By using primers that span exon-exon boundaries or large introns, no PCR product should be amplified from either the minus-RT control or the minus-template PCR control with Cells-to-Signal except with pseudogenes. Even if a primer set is used that amplifies genomic DNA, a difference in Ct value should still be observed between RT-plus and RT-minus.

If DNase treatment is absolutely needed for your experiment, use the Cells-to-cDNA II Kit (P/N AM1722, AM1723).

PCR products in the minus-RT control

Possible cause:
There is a report that thermostable DNA polymerases, including native Taq, can exhibit low-level intrinsic reverse transcriptase activity (Maudru and Peden, 1997). This could potentially cause an RT-PCR product to be made from a minus-RT control in the absence of contaminating DNA.

Solutions:
If PCR primers are designed to flank at least one large intron, then no PCR products from contaminating genomic DNA will typically be amplified with the recommended Cells-to-Signal cycling parameters.

Occasionally a processed pseudogene (introns spliced out) may be present in genomic DNA. If this is the case (as it is for actin), even primers that flank an intron will produce the same PCR product from both genomic DNA and cDNA. To ascertain if products of the RT-PCR are due to DNA contamination include both a minus-RT, and minus-template control with every reaction.

PCR products in the minus-template PCR control

Possible cause:
Bands in the minus-template PCR control indicate that DNA contamination of the sample occurred. Contamination of PCR reagents, pipettors, and benchtops with DNA is quite common.

Solutions:
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 bar-

rier 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 with fresh ones.

It is always a good idea to routinely include a minus-template negative control reaction with experimental PCRs. If minus-template controls routinely yield PCR products, more stringent steps may be taken to control contamination (see Yap, et al. 1994).

V. Appendix

A. References

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Pallansch L, Beswick H, Talian J, Zelenka P (1990) Use of an RNA folding algorithm to choose regions for amplification by the polymerase chain reaction, *Analytical Biochem*. **185**:57–62.

Sumida A, Yamamoto I, Zhou Q, Morisaki T, Azuma J (1999) Evaluation of induction of CYP3A mRNA using the HepG2 cell line and reverse transcription-PCR, *Biol. Pharm. Bull*. **22**:61–65.

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Yap EPH, Lo Y-MD, Fleming KA, and McGee JO'D (1994) False-Positives and Contamination in PCR. In: Griffen HG and Griffen AM (Eds.) *PCR Technology: Current Innovations*, CRC Press, p 249–258.

B. Quality Control

Functional testing

Components are functionally tested in Cells-to-Signal by producing cDNA from cultured cells, and amplifying hTBP from a serial dilution of the cDNA from 10–2500 cell equivalents. Also, the Control RNA is used in Cells-to-Signal, and the cDNA produced is subjected to qPCR using the Control RNA Primer Pair. All results are analyzed by real-time PCR.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

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

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

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

Protease testing

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

C. Safety Information

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 goggles, 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.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining the MSDS

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com) or telephone (650-554-2756; USA) your request, specifying the catalog or part number(s) and the name of the product(s). We will e-mail the associated MSDSs unless you request fax or postal delivery. Requests for postal delivery require 1–2 weeks for processing.

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