Cells-to-cDNA™ II Kit

Reverse Transcription without RNA Isolation

Part Number AM1722, AM1723

Cells-to-cDNA™ II Kit

(Part Number AM1722, AM1723)

Protocol

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I. Introduction

A. Background

The Cells-to-cDNA™ II Kit is designed to produce cDNA from mammalian cells in culture without isolating RNA. The cDNA produced is specifically intended for use in the polymerase chain reaction (PCR). RT-PCR is one of the main methods used for measuring mRNA levels from a small number of cells. Traditionally, the substrate for RT-PCR has been RNA isolated from the sample. RNA isolation is fairly time consuming, and with small samples, it can lead to loss of material. By eliminating most of the RNA isolation procedure using Cells-to-cDNA II, the RT-PCR analysis of a large number of samples becomes much faster and simpler.

Klebe et al., (1996) developed a strategy of adding placental RNase inhibitor to a crude cell lysate. Although successful, this method is limited because placental RNase inhibitor is only effective against members of the RNase A superfamily. There are many other types of cellular RNases that can contribute to RNA degradation that are not inhibited by placental RNase inhibitor. The Cells-to-cDNA II procedure inactivates all RNases present in cultured mammalian cells by heat treatment in the Cell Lysis II Buffer included with the kit.

Procedure overview

In the Cells-to-cDNA II Kit, a crude cell lysate is subjected to RT-PCR without purifying the RNA. Cells from tissue culture are washed in PBS and then heated in Cell Lysis II Buffer. This treatment has two important effects. First, it ruptures the cells, releasing the RNA into the Cell Lysis II Buffer. The heating step also inactivates endogenous RNases, protecting the RNA from degradation. Next the crude cell lysate is treated with DNase 1 to degrade genomic DNA, and the mixture is heated a second time to inactivate the DNase 1. At this point the cell lysate is ready for reverse transcription and PCR using either a one-step or two-step RT-PCR strategy.

Applications

The Cells-to-cDNA II Kit is well suited to the analysis of a large number of differentially treated cultures. For example, the regulation of an mRNA may be followed as cells are treated with increasing concentrations of a particular chemical (Sumida et al., 1999). Alternatively, cells may be treated with a panel of different drugs to screen for candidates that have the desired effect on a particular mRNA, or a time course can be followed (Su et al., 1997). Ambion® QuantumRNA™ Internal Standards are ideal for these types of quantification studies, because they enable the use of 18S rRNA as an internal standard. Cells-to-cDNA II products can be used in real-time PCR*, as shown in Figure 1, and the procedure lends itself to automation in 96-well plates.

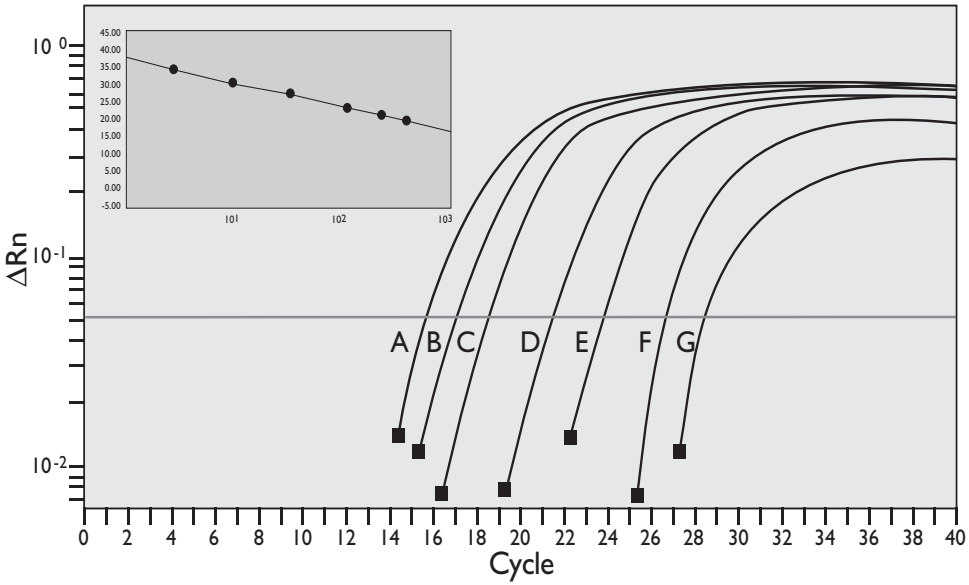

Figure 1. Real time RT-PCR of GAPDH using Cells-to-cDNA™ II

A serial dilution of HeLa cells was used as template in the Cells-to-cDNA II procedure. A sample of the cDNA produced (20% of the reaction product) was then used as a template for amplification of GAPDH in real-time PCR. A) 12,500 cell equivalents B) 6250 cell equivalents C) 2500 cell equivalents D) 500 cell equivalents E) 100 cell equivalents F) 20 cell equivalents G) 5 cell equivalents. The graph shows the fluorescence of each PCR product plotted against the PCR cycle number. ***Inset***: The standard curve of Ct value vs. cell concentration. The correlation was 0.99.

Of course, this procedure can also be used simply to amplify a particular sequence for cloning or as an efficient method for generating an RT-PCR template for samples consisting of very few cells (Figure 2).

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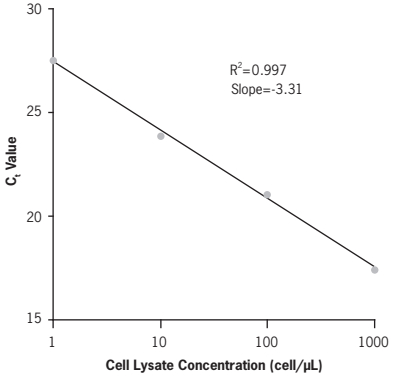

Figure 2. Linear Detection of CDC2 Using the Cells-to-cDNA™ II Kit

K562 cells were serially diluted and cell lysates were prepared using the Cells-to-cDNA II Kit according to the protocol to give the indicated lysate concentrations. For this experiment, 2 µL of each cell lysate was used as a template for amplification of CDC2 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-cDNA II Kit

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

- HeLa (Human)
- HeLa S3 (Human)
- K562 (Human)
- CHO (Chinese Hamster)
- COS-7 (African Green Monkey)
- J558 (mouse)
- MCF-7 (Human)

B. Materials Provided with the Kit and Storage Conditions

Storage at –20°C should be in a non frost-free freezer. Properly stored kits are guaranteed for 6 months from the date of shipment.

P/N AM1722 40 rxn	P/N AM1723 100 rxn	Component	Storage
40 mL	40 mL	1X PBS (pH 7.4)	–20°C*
4 mL	10 mL	Cell Lysis II Buffer	–20°C
80 µL	200 µL	DNase 1 (2 U/µL)	–20°C
200 µL	500 µL	10X RT Buffer	–20°C
40 µL	100 µL	M-MLV Reverse Transcriptase	–20°C
40 µL	100 µL	RNase Inhibitor (10 U/µL)	–20°C
160 µL	400 µL	dNTP Mix (2.5 mM each dNTP)	–20°C
80 µL	200 µL	Random Decamers (50 µM)	–20°C
80 µL	200 µL	Oligo(dT)$_{18}$ Primers (50 µM)	–20°C
50 µL	120 µL	Armored RNA® Control	–20°C*
80 µL	200 µL	Armored RNA® Primer Pair (10 µM ea)	–20°C
80 µL	200 µL	Endogenous Primer Pair (5 µM ea)	–20°C
1.75 mL	3.5 mL	Nuclease-free Water	any temp†

* The Armored RNA® Control and the 1X PBS may alternatively be stored at 4°C.
† Store Nuclease-free Water at –20°C, 4°C, or room temp.

C. Materials Not Provided with the Kit

- Compatible thermostable DNA polymerase

  Table 1. Compatible thermostable DNA Polymerases

Conventional thermostable DNA polymerases	Hot start thermostable DNA polymerases
Ambion® SuperTaq™ Polymerase	Sigma JumpStart Taq
ABI AmpliTaq®	Invitrogen Platinum® Taq
Roche Taq	Eppendorf HotMaster® Taq
Epicenter MasterAmp™ Tfl	Qiagen HotStarTaq

- PCR primers for the target of interest
  Verify that PCR primers function well with cDNA made from purified RNA before trying to use them with cDNA made from this kit.
- 10X PCR Buffer
- Thermal cycler for PCR
- Materials and equipment for analyzing DNA on agarose gels

D. Related Products Available from Applied Biosystems

*SuperTaq™ Polymerase P/N AM2050, AM2052	Thermostable DNA Polymerase (includes 10X buffers and dNTPs)
†SuperTaq™ Plus Polymerase 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.
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.

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II. Cells-to-cDNA II Procedure

A. Important Experimental Parameters

The Cells-to-cDNA II procedure was designed for mammalian cells grown in tissue culture. It was primarily tested with HeLa S3 cells grown in DMEM medium (Gibco BRL) supplemented with fetal bovine serum (FBS), but it has also been successfully used with other cells lines such as CHO, COS-7, J558, and MCF-7 cells. The cells should be grown using the conditions and treatments under study. Any type of culture vessel can be used. The parameters important for the success of the procedure are discussed below.

1. Cell lysis, RNase inactivation temperature

In step B.2 on page 10, it is crucial that the temperature of the sample itself reach 75°C. To achieve this, use a calibrated heating device, and preheat it well in advance of starting the procedure. Also, in some cases it will be necessary to set the temperature of the heating device above the target temperature to heat samples rapidly and hold them at 75°C. Some guidelines are provided below for different types of heating devices and sample containers:


NOTE

We recommend performing all heating steps in a thermal cycler, rather than in a dry heat block. For cells growing in 96-well plates, better results can be obtained by transferring the cell lysate into a PCR plate or into strip tubes, and performing all heating steps in a thermal cycler.

- Microcentrifuge tubes heated in a heat block or water bath: Use a recently calibrated device, and set it to 75°C. Since it is so important that the cell lysis and RNase inactivation temperature is at least 75°C, it is a good idea to measure whether a mock sample reaches 75°C using a thermometer with a microprobe. If necessary, increase the temperature setting of the heat source until the sample reaches and holds at 75°C.
- Thin wall, 0.5 mL PCR tubes should be heated in a thermal cycler set to 75°C.
- To use 96-well plates for optimization experiments, be aware that there may be as much as a 10–20°C temperature difference between the temperature setting of the heating device and the temperature of solutions in the wells of the plate. Set the heating device so that the ***contents*** of the 96-well plate will reach 75°C in ~10–12 min, and increase the incubation time in step II.B.2 on page 10 to 15 min. Turn on the heating device so that it will reach the target temperature before it is needed. For oven incubators, we recommend heating 96-well plates on an aluminum block placed (and preheated) inside the incubator.

2. Volume and temperature of Cell Lysis II Buffer

Use 100 µL ice-cold Cell Lysis II Buffer per sample.
It is important to lyse the cells in 100 µL of ice-cold Cell Lysis II Buffer. The Cell Lysis II Buffer should be ice-cold to protect the RNA from degradation; as soon as the cells are exposed to it they will lyse, and

RNases have minimal activity at 0°C. The 100 µL volume is recommended so that the temperature of the cell lysate will ramp to 75°C rapidly. Be sure that the lysate reaches a final temperature of 75°C for complete RNase inactivation.

3. Cell concentration

The maximum concentration of cells that can be used varies somewhat with the cell type. To obtain maximum sensitivity, the optimal cell concentration for HeLa cells is 2500 cells/µL Cell Lysis II Buffer (2.5×10^5 cells total). At higher concentrations, it may still be possible to amplify messages, but a less intense band may be produced compared to the optimal cell concentration. Determining the ideal cell concentration is crucial, because if the cell concentration is too high (typically >2500 cells/µL), then RNases in the sample may not be totally inactivated and/or the high concentrations of cellular components (debris) may inhibit reverse transcription.

Ambion strongly recommends doing a pilot experiment with the Armored RNA® Control to determine the ideal cell concentration for each of your cell types.

In a pilot experiment, a constant amount of the Armored RNA Control is added to a serial dilution of cells; the mixture is lysed and DNase treated, and the lysates are reverse transcribed. cDNA is then amplified using the Armored RNA Primer Pair. The highest cell concentration that produces an Armored RNA PCR product equivalent to the amount amplified in the absence of cells is considered the optimal cell concentration. Instructions for doing this pilot experiment are included in the protocol alongside the instructions for experimental samples.

4. PCR primer selection

For the most efficient design of PCR primers for RT-PCR applications, use primer design software. These programs can identify and avoid primers that are likely to generate unwanted side products, and they provide useful information about primer annealing temperature and other cycling parameters. 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 et al., 1990). It is also useful to choose primers that span an exon-exon boundary in the target mRNA to minimize amplification from genomic DNA, or to distinguish PCR products amplified from genomic DNA.

5. Test the PCR reagents on cDNA from purified RNA

Make sure the PCR works well on cDNA reverse transcribed from purified RNA from the same cell line before trying to use those primers and reagents on cDNA from Cells-to-cDNA II. Remember that in a typical Cells-to-cDNA II reaction, the RNA from ~12,500 cells will be reverse transcribed; this will be ≤50 ng of total RNA. Try doing RT-PCR on a titration of purified RNA from your source. If a product can be

amplified from ~50–200 ng RNA, there should be no problem amplifying the same fragment from Cells-to-cDNA II reactions. Otherwise, work on optimizing the PCR with purified RNA as template before trying to amplify cDNA from Cells-to-cDNA II (e.g. denaturation temperature, cycle times, primer sequence, etc.).

6. One-step or two-step RT-PCR

Traditionally reverse transcription (RT) and PCR are done as two separate sequential reactions. Using the Cells-to-cDNA II Kit, however, a one-step RT-PCR can be used to save time and to facilitate higher throughput. In one-step RT-PCR, the reverse transcription and PCR reagents are added to the template in a single vessel at the same time. Combining the RT and PCR into a single reaction greatly reduces the amount of time it takes to go from a cell lysate to PCR product. The Cells-to-cDNA II Kit contains reagents for reverse transcription, but thermostable DNA polymerase (available from Ambion®) and gene specific primers must be purchased separately. Both one- and two-step RT-PCR procedures are included in section II.C starting on page 11.

B. Cell Lysis, DNase I Treatment and Inactivation

1. Detach cells (if necessary) and wash once in cold PBS

The main purpose for washing the cells is to remove the serum in the medium because it contains ribonucleases and inhibitors of reverse transcription. The type of vessel in which the cells were grown and the adherence property of the cells will dictate the method used for washing. Use the method commonly employed in your lab to wash cells in cold PBS, or follow the instructions provided below for adherent cells grown in a 96-well plate (step 1a on page 8), Adherent cells grown in other vessels (step 1b on page 9) or cells in suspension (step 1c on page 9). It may be possible to omit the washing step entirely if the growth medium has very little serum or if only a few cells will be added to the Cell Lysis II Buffer. (This can be determined using the Armored RNA control.)

1a. Adherent cells grown in a 96-well plate

a. Count or estimate the number of cells. Make sure that each well doesn't contain more than 100,000 cells because that would result in a final cell concentration that could inhibit RT-PCR. If your cells grow to >100,000 cells per well, we recommend determining the optimal cell concentration by doing the pilot experiment described below.

b. Aspirate the medium from the well.

c. Add 0.2 mL of cold 1X PBS to the well.

d. Aspirate the PBS from the well.

The cells are now ready for lysis (step 2 on page 10).

1b. 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. Some cell types can be removed by simply scraping them 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.

a. Detach the cells (use the method routinely followed in your tissue culture facility for the cell type).
   If trypsin is used, inactivate it before proceeding (typically this is done by rinsing in 1X PBS and resuspending in culture medium containing serum).

b. Resuspend cells in culture medium and count or estimate the number of cells.

c. ***Pilot experiment:*** Aliquot different amounts of cells into RNase-free tubes and place on ice (cells should be in culture medium at this step—not in Cell Lysis II Buffer). We recommend testing from about 10^4 cells to ~10^6 cells in increments of 2- to 5-fold, (i.e. 10^4, 5 x 10^4, 10^5, 5 x 10^5, 10^6 cells). Be sure to include a control with no cells.
   Subsequent experiments: Place an aliquot containing the optimal number of cells (as determined in the pilot experiment) in a clean microfuge tube on ice.

d. Rinse cells at least once in cold (4°C) 1X PBS.

e. Pellet the cells at 4°C, discard the 1X PBS, and replace in ice. To pellet the cells, use the centrifugal force and time typical for the particular cell line; this is often ≤1200 x g for ~5 min.
   The cells are now ready for lysis (step 2 on page 10).

1c. Cells grown in suspension

a. Count or estimate the number of cells. Cells grown in suspension can be counted directly in their growth medium using a hemocytometer.

b. ***Pilot experiment:*** Aliquot different amounts of cells into RNase-free tubes, and place on ice (cells should be in culture medium at this step—not in Cell Lysis II Buffer). We recommend testing from about 10^4 cells to ~10^6 cells in increments of 2-fold to 5-fold, (i.e. 10^4, 5 x 10^4, 10^5, 5 x 10^5, 10^6 cells). Be sure to include a control with no cells.
   Subsequent experiments: Place an aliquot containing the optimal number of cells (as determined in the pilot experiment) in a clean microfuge tube on ice.

c. Pellet the cells at 4°C, discard the growth medium (e.g. by aspiration), and replace them on ice. To pellet the cells, use the centrifugal force typically used for the particular cell line; this is often ≤1,200 x g for ~5 min.

d. Add ~1 mL cold (4°C) 1X PBS and flick the tube gently to resuspend the cell pellet.

e. Re-pellet the cells, remove the 1X PBS by aspiration, and replace the cells in ice.
The cells are now ready for lysis (step 2 below).

2. Add 100 µL ice-cold Cell Lysis II Buffer, mix, and incubate 10 min at 75°C

This step lyses the cells, releasing their RNA. It also inactivates RNases that are either released from the cells or that are present in any remaining growth medium. It is very important that the samples are heated to at least 75°C, see section A.1 on page 6 for recommendations on using different heating devices for this step.

Pilot experiment:

a. Mix 1 µL Armored RNA Control per 100 µL of Cell Lysis II Buffer. You will need 100 µL of solution per sample.

b. Add 100 µL ice-cold Cell Lysis II Buffer + Armored RNA Control to each sample, mix by vortexing or pipetting, and leave on ice until Cell Lysis II Buffer has been added to all samples. Include a sample that has no cells, only the Armored RNA Control. This sample will be the standard to which the samples with cells will be compared.

c. Immediately transfer to a heating device, and incubate for 10 min at 75°C—for 96-well plates, incubate 15 min.

Subsequent experiments:

a. Add 100 µL ice-cold Cell Lysis II Buffer to cells on ice and mix by vortexing or pipetting. Keep the experiment on ice until Cell Lysis II Buffer has been added to all samples.

b. Once Cell Lysis II Buffer has been added to all the samples, immediately transfer to a heating device, and incubate for 10 min at 75°C—for 96-well plates, incubate 15 min.

3. Cool the sample on ice

Remove the sample from the heat source and place on ice.

4. Add 2 µL DNase I per 100 µL Cell Lysis II Buffer

The final DNase I concentration should be 0.04 U/µL.

Mix the solution ***thoroughly*** by vortexing gently, centrifuge briefly to bring the solution to the bottom of the tube or plate.

5. Incubate at 37°C for 15 min

Incubate the DNase I reaction at 37°C for 15 min to degrade the genomic DNA in the sample. This is especially important if the primer pair for PCR does not span an exon-exon boundary or if there are pseudogenes for the target in the genome. Otherwise, PCR products generated from the genomic DNA will be indistinguishable from those amplified from cDNA.

6. Inactivate the DNase at 75°C for 5 min

It is important to inactivate the DNase by heating to 75°C for 5 min because otherwise it would degrade the products of reverse transcription.

NOTE

Lysates made from ≥2.5 x 10^4 cells can be stored at –20°C for periods up to 1 week, or at –80°C for up to 2 months.
Lysates made from <2.5 x 10^4 cells should be used for RT-PCR immediately.

C. RT-PCR

One- or two-step RT-PCR

Procedures for both two-step and one-step RT-PCR are shown below. We recommend using the two-step procedure for initial experiments with new targets and/or cells. If two-step RT-PCR produces easily detectable products from most or all of the samples, then it is feasible to try one-step RT-PCR. The major advantage of one-step RT-PCR is that it is considerably quicker than the two step strategy.

C.I. Two-Step RT-PCR

1. Primers for reverse transcription

Type of primers

Generally 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, for example a reverse (antisense) PCR primer

The choice of first strand primer is mostly based on user preference. The greatest yield of cDNA is usually obtained by using short random primers, however, this yield advantage is often no longer seen at the end of the PCR. In fact the yield of specific product after the PCR step may be the same using either random primers or oligo(dT). When template is limiting, final RT-PCR yield may be somewhat higher when the first-strand reaction is primed with random primers as opposed to oligo(dT) or gene-specific primers (see Innis et al., Chapter 3, for further discussion).

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 primers, oligo(dT), and the reverse PCR primer.

Concentration of primers for RT

The concentration of both the Random Decamers and the Oligo(dT) Primers supplied with the Cells-to-cDNA II Kit is 50 µM, yielding a final concentration of 5 µM in the RT reaction. If a gene specific primer is used to prime the reverse transcription, its final concentration should be 0.25–5 µM (Innis et al, Chapter 3).

2. Heat denaturation of the RNA template

The template RNA is usually heated just before reverse transcription in two-step RT-PCR experiments. This denatures any secondary structure that could impede the ability of the enzyme to make a full-length copy of the template RNA. Since heating can reduce or inactivate reverse transcriptase activity, the reaction is typically assembled in two parts. There is some flexibility in choosing which components to add before or after the heat denaturation; the idea is to heat denature the RNA in the lysate, and to avoid heat denaturing the heat-labile RT reaction components. The reaction volume at the heating step should be at least ~5 µL; otherwise, the contents of the tube may evaporate.


NOTE

It is often possible to omit preheating of the lysate prior to assembling the reaction. Good yields of the expected product can often be obtained in reverse transcription reactions, even for G+C rich targets, without preliminary heating of the sample.

3. Procedure for reverse transcription

a. Assemble the following in a nuclease-free microfuge tube, then mix gently and centrifuge briefly:


NOTE

When multiple reactions are carried out, cocktails of the RT reagents can be prepared to minimize labor.

Amount	Component
5–10 µL	cell lysate (RNA)
4 µL	dNTP Mix
2 µL	first-strand primers*
to 16 µL	Nuclease-free Water

* Use 2 µL of the supplied Random Decamers or Oligo(dT)$_{18}$ Primers. If you use a gene specific primer, its final concentration should be 0.25–5 µM (in 20 µL final reaction volume).

Pilot experiment: Use 5 µL cell lysate, and use random primers.

Minus-RT control: Because the cell lysate may have contaminating genomic DNA even after the DNase treatment, it is important to do a minus-reverse transcriptase control to demonstrate that the tem-

plate for the PCR product was cDNA, and not genomic DNA. Typically, the control is a mock reverse transcription, containing all the RT reagents except the Reverse Transcriptase.

b. (optional) Heat 3 min at ~70°C.

c. Place reaction on ice for 1 min; centrifuge tube briefly and replace on ice.

d. Add the remaining RT reagents, then mix gently and centrifuge briefly:

Amount	Component
2 µL	10X RT Buffer
1 µL	M-MLV Reverse Transcriptase (for the minus-RT control add water here)
1 µL	RNase Inhibitor

e. Incubate at 42°C for 15–60 min.

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

g. Store reaction at –20°C or proceed to amplification step.

4. PCR amplification

a. Assemble the components on ice in thin-walled microcentrifuge tubes and mix gently:

IMPORTANT

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

Amount	Component
1–5 µL	RT reaction (for the minus-template control use water here)
5 µL	10X PCR Buffer
4 µL	dNTP Mix (2.5 mM each)
to 47 µL	Nuclease-free Water
2 µL	PCR primers (mixture of 10 µM each primer)
2 unit	thermostable DNA polymerase

Pilot experiment: Use 5 µL of the RT reaction, and use 2 µL of the Armored RNA Primer Pair.

Negative Controls: Include two negative controls. One is the minus-RT control from the previous step, and the other is a minus-template PCR. The minus-template PCR should have all the

PCR components, with water substituted for the RT reaction aliquot. This control will verify that none of the PCR reagents are contaminated with DNA.


NOTE

Thermal cyclers with very short ramp times may require slightly longer incubation times, whereas machines with virtually no ramp time, such as Stratagene's RoboCycler®, typically require 1 min at each temperature in the cycle for good results.

b. Cycle as follows:

denature:	94°C – 2 min
35 cycles:	94°C – 30 sec
	annealing temp.* – 30 sec
	72°C – 30 sec
final extension:	72°C – 5 min

* ***Pilot Experiment:*** Use 60°C annealing temperature.
Subsequent experiments: Start with the annealing temperature suggested by your primer design software. An annealing temperature of ~55°C used with the cycling times shown is often a reasonable starting point, but the optimal temperature and cycling times for your primer and template combination may have to be determined empirically.

C.II. One-Step RT-PCR

1. Negative Controls

Include two negative controls. One is the minus-RT control with all the reaction components except the reverse transcriptase, and the other is a minus-template control where water is used instead of cell lysate.

2. Assemble the reaction

Assemble the following on ice in thin-walled microfuge tubes, mix gently and then centrifuge briefly to collect the contents at the bottom of the vessel:

Amount	Component
1–5 µL	cell lysate (for the minus-template control add Nuclease-free Water)
2.5 µL	10X RT Buffer
4 µL	dNTP Mix (2.5 mM each)
1 µL	RNase Inhibitor
1 µL	M-MLV Reverse Transcriptase (for the minus-RT control add Nuclease-free Water)
1 µL	PCR primers (mixture of 10 µM each primer)
2 unit	thermostable DNA polymerase
to 25 µL	Nuclease-free Water

Pilot experiment: Use 5 µL cell lysate, and use 1 µL of the Armored RNA Primer Pair as the PCR primers.

3. Cycle as follows:

One-step RT-PCR cycling program

reverse transcription:	42°C – 15 min
denature:	94°C – 2 min
35 cycles:	94°C – 30 sec
	annealing temp.* – 30 sec
	72°C – 30 sec
final extension:	72°C – 5 min

* ***Pilot Experiment:*** Use 60°C annealing temperature.
Subsequent experiments: Start with the annealing temperature suggested by your primer design software. An annealing temperature of ~55°C used with the cycling times shown is often a reasonable starting point, but the optimal temperature and cycling times for your primer and template combination may have to be determined empirically.

D. Analysis of the Data

1. Run PCR products on an agarose gel

To analyze the RT-PCR, run an aliquot (typically 10–20 µL) on a native agarose gel in the presence of ethidium bromide, and visualize the product under UV light. Any typical gels for DNA analysis can be used. The amount and type of agarose used should be determined based on the expected size of the reaction products. Generally 2% high resolution agarose in 1X TBE works well. We typically stain nucleic acids by adding ethidium bromide (0.5 µg/mL) to the running buffer.

The remaining RT-PCR can be stored at –20°C. If desired, the product can be further purified, for example by gel electrophoresis and electroelution, column filtration, organic extraction, or alcohol precipitation. For many purposes, for example digestion with most common restriction enzymes, or as transcription template, further purification of the RT-PCR product is not required.

Negative controls
Both the minus-RT and the minus-template negative controls should not produce visible PCR products. If bands appear in these lanes, see section III.C on page 30, for troubleshooting suggestions.

2. Analysis of the pilot experiment to determine the optimal cell concentration

The pilot experiment compares amplification of the Armored RNA Control alone with its amplification in the presence of cell lysate from increasing concentrations of cells. When looking at the gel from the pilot experiment, identify the lane from the sample containing only the Armored RNA Control (with no cells). The optimal cell concentration for the type of cells tested will be the concentration used in the lane that most closely resembles the Armored RNA Control-only lane. This will typically not be the highest number of cells (containing the largest amount of template RNA). At higher cell concentrations, the PCR

product (147 bp) may be less intense than the product at lower cell concentrations. This reduced amplification is attributable to two possible causes.

- At higher cell concentrations, the RNase inactivation could have been incomplete, causing some degradation of the control RNA.
- Also, at higher cell concentrations, the cell lysate could have inhibited the reverse transcriptase.

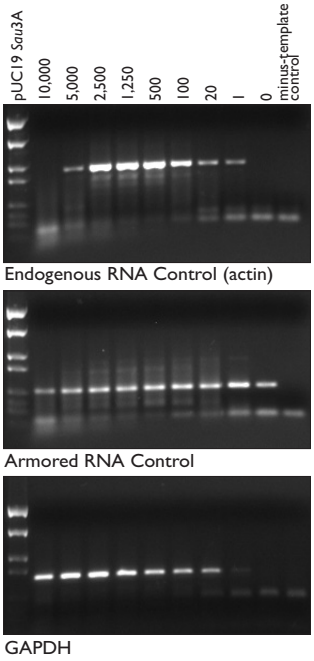

Figure 3. Pilot Experiment to Determine the Optimal Cell Concentration for HeLa Cells

The indicated amounts of HeLa cells per µL Cell Lysis II Buffer were used in the Cells-to-cDNA II pilot experiment to determine the optimal cell concentration. Looking at the center gel showing amplification of the Armored RNA Control, we see that at about 10,000 cells/µL Cell Lysis II Buffer the amplification of the Armored RNA Control starts to suffer. The top and bottom gels provide an idea of the cell concentration needed for amplification of the message being studied. Using the Endogenous Primer Pair, amplification of the Endogenous RNA Control is optimal with 100–2500 cells/µL Cell Lysis II Buffer. Using primers for GAPDH, however, amplification is still very strong even with 10,000 cells/µL Cell Lysis II Buffer. This experiment illustrated why it is useful to do a pilot experiment with each set of PCR primers to identify the best cell concentration for the most sensitive RT-PCR.

III. Troubleshooting

A. Positive Control Reactions

1. Armored RNA® Control

Armored RNA Control

The Cells-to-cDNA II Kit includes an Armored RNA Control (US patents issued and pending). It is a control RNA sequence protected by a multimeric formation of protein dimers to produce a bacteriophage-like particle. The RNA is thereby "Armored", and completely protected from RNase degradation (Pasloske et al., 1998, DuBois et al., 1999). By heating the Armored RNA to 75°C, the protein coat denatures, releasing the RNA, which is can then serve as a template for reverse transcription. The Armored RNA Control is ideal for the Cells-to-cDNA II Kit because it can be used to monitor the efficiency of the RNase inactivation step, and any inhibitory effects of the cell lysate on reverse transcription.

Armored RNA Primer Pair

An Armored RNA Primer Pair is also included with this kit to amplify the Armored RNA Control. ***The Armored RNA should generate a 147 bp PCR product*** when amplified with the Armored RNA Primer Pair.

2. Routine use of the Armored RNA Control

The Armored RNA Control may be added to the Cell Lysis II Buffer used to resuspend every test sample as a matter of routine to help troubleshoot problems if they occur. (Use 1 µL Armored RNA Control for every 100 µL Cell Lysis II Buffer.) If no PCR product is generated using the experimental PCR primers, then try to amplify the Armored RNA Control with the Armored RNA Primer Pair from another aliquot of the treated lysate. Use the RT-PCR conditions outlined in step 3.d on page 18. ***The Armored RNA should generate a 147 bp PCR product.***

If the 147 bp control PCR product is amplified, it indicates that the cell lysis and RT-PCR worked properly.

3. Use of the Armored RNA Control to test RT-PCR reagents

Use the Armored RNA Control alone (without any cell lysate) to verify that the RT-PCR is functioning properly.

a. Add 1 µL of the Armored RNA Control to 100 µL of Cell Lysis II Buffer.

b. Heat the mixture to 75°C for 10 min.

c. Continue with the DNase treatment and inactivation as described in steps 4–6 on page 11.

d. Use 5 µL of the Cell Lysis II Buffer + Armored RNA as template in either one-step or two-step RT-PCR following the instructions in section C.I starting on page 11 or C.II starting on page 14.
   - For two-step RT-PCR, use Random Decamers to prime the RT, and use 5 µL of the RT reaction as template for the PCR.
   - Use the Armored RNA® Primer Pair for the PCR
   - Annealing temperature for the PCR should be 60°C
   - Include minus-RT and minus-template controls in the experiment.

e. Fractionate 10–20 µL of the PCRs on a 2% agarose gel.
   The Armored RNA should generate a 147 bp PCR product.

   There should be no product in either of the two negative control reactions.

   If the 147 bp Armored RNA PCR product is not seen, then there may be a problem with one or more of the RT or PCR components.

NOTE

The concentration of the Armored RNA Control and the RT-PCR conditions specified for this control reaction were established using the Perkin Elmer 9600 thermal cycler at Ambion and SuperTaq DNA Polymerase, such that the yield of the PCR product would generate a signal in the linear range by UV fluorescence. If a PCR product is not generated using your thermostable DNA polymerase and thermocycler, then it may be necessary to modify the cycling parameters, or increase the amount of Armored RNA Control added to the Cell Lysis II Buffer.

4. Endogenous RNA Control

An Endogenous Primer Pair is provided with the kit to amplify actin cDNA reverse transcribed from human, mouse, or rat cell lysates. These primers will also amplify actin pseudogenes in genomic DNA, therefore the lysate must be treated with DNase before reverse transcription. Besides their utility as a control for amplification of an endogenous message, the Endogenous Primer Pair can be used to monitor the efficiency of the DNase digestion. We also recommend including the Armored RNA Control in experiments using the Endogenous Primer Pair. This simplifies distinguishing between problems with the RNA from the cells, and problems with the RT-PCR itself.

If your cells are derived from human, mouse or rat, use the Endogenous Primer Pair as follows:

a. Count the cells and wash them once in cold PBS as described in step 1 starting on page 10.

b. Using 5000 cells, follow the instructions in steps 2–6 starting on page 11 for the pilot experiment.

c. Use 5 µL of the lysate as template in either one-step or two-step RT-PCR following the instructions in section C.I starting on page 11 or C.II starting on page 14.
   - For two-step RT-PCR, use Random Decamers to prime the RT, and use 5 µL of the RT reaction as template for the PCR.
   - Use the Endogenous Primer Pair for the PCR
   - Annealing temperature for the PCR should be 60°C
   - Include minus-RT and minus-template controls in the experiment.

d. Analysis of results
   Analyze results by running 10–20 µL of the PCRs on an ethidium bromide stained agarose gel.

   The Endogenous Primer Pair will generate a 294 bp PCR product.

   PCR products that are not 294 bp may also be seen due to isoforms of actin. The minus-RT control should not produce a PCR product—if one is seen, then the DNase treatment may not have been sufficient. In subsequent experiments try the following to improve the DNase digestion:
   - Double the amount of DNase used, in other words use 2 µL DNase/50 µL Cell Lysis II Buffer
   - Increase the DNase incubation from 15 to 30 min

B. No PCR Product or Unexpected PCR Products

1. The PCR requires optimization

a. 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.

b. 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.

2. The sample was not kept cold enough prior to RNase inactivation

It is very important to keep samples in ice at all times before the heat treatment that serves to inactivate cellular RNases (step II.B.2 on page 10). Keeping samples very cold minimizes the activity of these RNases until the 75°C incubation.

3. RNase in the sample was not completely inactivated

The cells plus Cell Lysis II Buffer were not incubated at 75°C
It is very important that the samples in Cell Lysis II Buffer reach 75°C quickly in step II.B.2 on page 10 to effectively inactivate cellular RNases. If you are getting no PCR product, or unexpected PCR products, consider measuring the temperature of the solution inside the reaction vessels during the 75°C heat treatment to make sure that the reactions reach 75°C.

Cell concentration in the Cell Lysis II Buffer was too high
Using too many cells in the Cells-to-cDNA II procedure can give poor results. Try fewer cells, and/or compare the cell number used in the failed experiment to 5–10 fold fewer cells using the Armored RNA Control as an indicator of RT-PCR efficiency. This procedure is described throughout section *II. Cells-to-cDNA II Procedure* under the heading ***Pilot experiment***.

4. Cell lysate contains inhibitors of RT-PCR

Try fewer cells, and/or repeat the control experiment as described throughout section *II. Cells-to-cDNA II Procedure* under the heading ***Pilot experiment***.

5. 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 doesn't 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 as described in section II.A.5 on page 7. If the amplification does not give good results using cDNA from purified RNA, it will not work with cDNA from Cells-to-cDNA II.

b. Verify that the Cells-to-cDNA II procedure is working by including an aliquot of Armored RNA Control in the sample as described in section *III.A.2. Routine use of the Armored RNA Control* on page 17. Also, if your cells are derived from human, mouse or rat, try to amplify actin using the Endogenous Primer Pair (see section III.A.4 on page 18). If you are able to amplify the Armored RNA Control and actin, then it is possible that the RNA of interest is undetectable with this procedure.

6. 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 would resolve this question. Alternatively, design primers to a unique region of the transcript (e.g. the 3' untranslated region), and try these in the PCR.

7. The RT reaction or PCR components are not working

Check the reaction components by doing the positive control reaction described in section *III.A.3. Use of the Armored RNA Control to test RT-PCR reagents* on page 17.

C. RT-PCR Products in the Negative Control(s)

No PCR product should be amplified from either the minus-RT control or the minus-template PCR control.

1. PCR products in the minus-RT control

Possible causes:

If PCR products are produced by the minus-RT control, it is probably because the DNase treatment was not effective, or possibly because the sample became contaminated with DNA after the DNase treatment. (DNA contamination after the DNase treatment is unlikely unless the contamination is introduced by the PCR reagents. This would also show up in the minus-template PCR.)

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:

The DNase treatment (step 4 on page 10) can be made more rigorous by using twice the DNase 1 (in other words use 2 µL DNase 1 for every 50 µL of Cell Lysis II Buffer for a final concentration of 0.08 U/µL) or by increasing the incubation time to 30 min.

If PCR primers are designed to flank at least 1 intron, then PCR products from contaminating genomic DNA will typically be a different size than products from cDNA. Occasionally a processed pseudogene (introns spliced out) may be present in genomic DNA. If this is the case (as it is for actin – the Endogenous Primer Pair target), even primers that flank an intron will produce the same PCR product from both genomic DNA and cDNA. One way to prove that products of the RT-PCR are due to DNA contamination is to include both minus-RT and minus-template control reactions routinely.

2. 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. Unfortunately the only way to remedy contaminated reagent(s) is to replace them with uncontaminated ones.

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 de-contamination product. Use barrier tips to pipette PCR reagents, and store completed PCRs in a different location than the PCR reagents.

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).

IV. Appendix

A. References

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B. Safety Information

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

IMPORTANT

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

To obtain Material Safety Data Sheets

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

Chemical safety guidelines

To minimize the hazards of chemicals:

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

C. Quality Control

Functional testing

Components are functionally tested by producing cDNA from cultured cells, and amplifying hTBP from a serial dilution of the cDNA from 10–1500 cell equivalents. Also, 1 µL of the Armored RNA Control is used in the Cells-to-cDNA II procedure and the cDNA produced is subjected to PCR using the Armored 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 25 ng labeled RNA and analyzed by PAGE.

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

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

Protease testing

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