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AmpEasy™ Cells qRT-PCR Kit (For Probe System w/ ROX)

Description

AmpEasy™ Cells qRT-PCR Kit (For Probe System w/ ROX) is designed for high-speed preparation of first-strand cDNA and real-time PCR amplification directly from cultured cells without RNA purification. By using AmpEasy™ Cells qRT-PCR Kit (For Probe System w/ ROX), RNA in the cell lysate can be directly converted to cDNA and subsequently analyzed using the real-time PCR reagents included in the kit. The whole process, from cells to cDNA and from cDNA to real-time PCR, can be completed in less than 1.5 hours. AmpEasy™ Cells qRT-PCR Kit (For Probe System w/ ROX) accelerates and streamlines real-time PCR analysis of cultured cells. This allows analysis of a large number of differentially treated cultures much faster and simpler.

Features

- High-speed preparation of first-strand cDNA directly from cultured cells without RNA purification.
- No RNA purification is required, minimizing pipetting tasks and saving plenty of time.
- Just less than 1.5 hours, from cells to cDNA and from cDNA to real-time PCR can be completed.
- Highest cDNA yields of specific and long cDNA synthesis (~2.1KB).
- Optimized for use with any real-time PCR cycler and sequence-specific probes.

Applications

- Ideal for performing reverse transcription reactions and real-time PCR on small number of cells.
- Ideal for analysis of large numbers of differentially treated cultures.
- Gene-expression analysis
- Reproducible results in high-throughput analysis

Quality Control

Specificity and reproducibility of AmpEasy™ Cells qRT-PCR Kits (For Probe System w/ ROX) are tested in reproducibility assay: parallel 20µl reactions containing 2ul of human total RNA from embryonic kidney cell lysate and 0.5 µM primers, specific for d(T)18. After 35 cycles, B2M transcript is detected. The length of cDNA achieved is verified as 248 bp by electrophoresis and DNA sequencing. Tenfold serial dilution (10^9~10^{10}) of cDNA were amplified using primers specific to the NNV gene. Triplicate reactions at each concentration were amplified along with no-template controls. Standard curve is r=0.995, efficiency=94.8% and standard deviation of Ct<1.0.

Shipping and Storage Conditions

AmpEasy™ Cells qRT-PCR Kit (For Probe System w/ ROX) is shipped on dry ice. Buffer W should be stored at room temperature (15–25℃). All other com ponents of AmpEasy™ Cells qRT-PCR Kit (For Probe System w/ ROX) should be stored immediately upon receipt at -20℃ in a constant temperature freezer and protected from light. Avoid repeated freeze–thaw cycles. With proper storage and handled correctly, AmpEasy™ Cells qRT-PCR Kit (For Probe System w/ ROX) can be stored for up to 12 months without showing any deduction in performance and quality.

Product Components and Storage Conditions

Cat. No.:	Storage Temp.	RCP050	RCP100
Product Name:		AmpEasy™ Cells qRT-PCR Kit	
Size:		50 preps	100 preps
Buffer Set (part 1 of 3):			
Buffer W	Room Temp.	25 ml	50 ml
Buffer C	-20℃	2.5 ml	5 ml
Reverse-Transcription Set (part 2 of 3):			
RealScript™ Reverse Transcriptase#	-20℃	100 ul	200 ul
2X First-Strand Reaction Mix*	-20℃	0.5 ml	1 ml
Oligo (dT) primer (10 µM)	-20℃	50 ul	100 ul
RNase-Free Water	-20℃	1 ml	1 ml
Real-Time PCR Set (part 3 of 3) :			
2X RealSens™ Real-Time PCR Mastermix	-20℃	625ul	1.25ml
RNase-Free Water	-20℃	1ml	2ml

Contains RNase inhibitor.

* Includes Mg^{2+} and dNTPs.

Principle and procedure

AmpEasy™ Cells qRT-PCR Kit (For Probe System w/ ROX) procedure comprises only 3 steps: cell wash, cell lysis, and two-step qRT-PCR.

Cell Wash

Cultured cells are briefly washed with Buffer W to remove cell-culture medium, extracellular material released by living cells, and intracellular material released by any dead, lysed cells. Removal of extracellular contaminants is important, since they can interfere with reverse transcription and subsequent quantification by real-time RT-PCR.

Cell Lysis

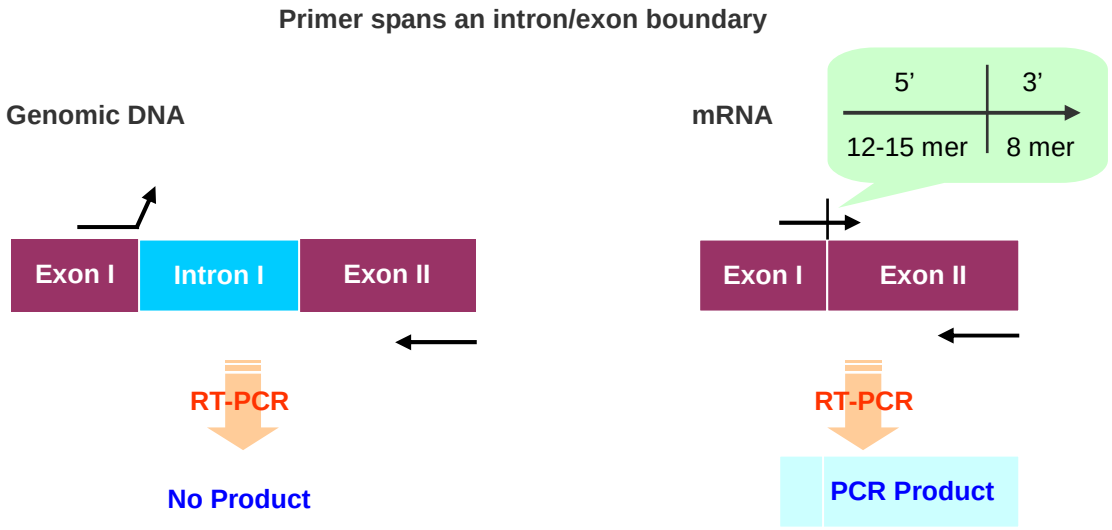
After the wash with Buffer W, the cultured cells are then lysed for 5 minutes using Buffer C. This buffer also stabilizes the cellular RNA and blocks inhibitors of reverse transcription. This allows efficient synthesis of first-strand cDNA from RNA that accurately reflects the in vivo gene expression profile. When human cultured cells are analyzed, the optimal cell concentration is $5x10^4$ cells (50 µl of Buffer C is required).

Reverse Transcription

After cell lysis, the cell lysate is ready for reverse transcription using Reverse-Transcription Set included in the kit. The RT reaction takes place at 42℃ and is then inactivated at 95℃. No additional steps for RNA denaturation, primer annealing, and RNase H digestion are required in the procedure. The cDNA produced is ready to be used in the subsequent PCR.

Primer Design

Since removal of genomic DNA is not included in AmpEasy™ Cells qRT-PCR Kit (For Probe System w/ ROX), genomic DNA will be amplified if inappropriate primer or probe is used. Detection of contaminating genomic DNA can be eliminated with specially designed primers as below.



Protocol

Important notes before starting:

1. Optimal cell concentration is $5x10^4$ cells. Suggested concentration is $1x\ 10^4$ cells to $1x\ 10^5$ cells.
2. If the expected PCR product size is larger than 500 bp, it is recommended to include 0.5 µl RNase inhibitor (40 unit/µl) in 50 µl of the Buffer C. (Refer to Cat. No. YRI001 RNAarmor™ RNase Inhibitor)

3. Do not add RNase inhibitor and dNTPs to the reverse-transcription reaction. RNase inhibitor and dNTPs are included in RealScript™ Reverse Transcriptase and 2X First-Strand Reaction Mix respectively.
4. Additional items to be prepared by the users: 1.5ml RNase-free plastic tubes, ice, heating block or water bath (capable of reaching 95℃), vortexer and micro centrifuge.

Step 1: Cell Wash

1. Transfer the cells (5 x 10^4) to a 1.5 ml microcentrifuge tube and harvest by centrifugation for 5 minutes at 4,000 rpm at room temperature.
2. Remove the supernatant completely and resuspend the cells in 500 µl of Buffer W.
3. Centrifugation for 5 minutes at 4,000 rpm at room temperature.

Step 2: Cell Lysis

1. Remove the supernatant completely and resuspend the cells in 50 µl of Buffer C.
2. Vortex 5 seconds and incubate at room temperature for 5 minutes.

Step 3: Two-Step qRT-PCR

Reverse Transcription

1. Thaw and add the following components to a sterile microtube on ice:

Component	Volume/ Reaction
Reverse-Transcription Set	
RealScript™ Reverse Transcriptase	2 µl
2X First-Strand Reaction Mix	10 µl
Oligo (dT) primer (10 µM)	1 µl
RNase-Free Water	5 µl
Template RNA	
Cell Lysate	2 µl
Total Volume	**20 µl**

2. Reverse Transcription: Incubate the mircrotube at 42℃ for 5 minutes.
3. Inactivation: Incubate the mircrotube at 95℃ for 10 minutes.
4. Store reverse-transcription reactions on ice and proceed directly with real-time PCR. For long-term storage, store reverse-transcription reactions at –20℃.

Real-Time PCR

Our protocol is for a reaction size of 25ul. This protocol serves only as a guideline for real-time PCR amplification. Optional reaction conditions may vary and must be individual determined.

Notes: Use disposable tips containing hydrophobic filters to minimize cross-contamination.

1. Thaw and add following components to a sterile microtube on ice:

Component	Volume/ Reaction	Final Concentration
2X RealSens™ Real-Time PCR Mastermix	12.5 µl	1X
Forward Primer (10µM)	0.75µl	0.3~0.6µM
Reverse Primer (10µM)	0.75µl	0.3~0.6µM
Probe	variable	0.2~0.4uM
RNase-Free Water	Add to 23.0µl	

2. Mix above components thoroughly by pipetting up and down and dispense the 23µl of mixture into PCR tubes or plates.
3. Add 2µl of the DNA or cDNA and mix carefully by pipetting up and down.
4. Suggested Real-Time Cycler Conditions. The temperature of X℃ should be above the Tm of Primer dimmers, but approximately 3℃ below the Tm of the specific PCR product. (Tm dimmer < X < Tm product).

For length of target gene shorter than 300 bp:

Segment	Number of Cycles	Temperature	Time
1	1	95℃	10 minutes
2	40~45	95℃	15 seconds
		X℃*	30 seconds
3	1	4℃	∞
* X: optimal annealing temperature is depending on user's primer sequences.			

For length of target gene longer than 300 bp:

Segment	Number of Cycles	Temperature	Time
1	1	95℃	10 minutes
2	40~45	95℃	15 seconds
		X℃*	25 seconds
		72℃**	10 seconds
3	1	4℃	∞
* X: optimal annealing temperature is depending on user's primer sequences. ** It takes around 1 minute for amplifying 1kb product at 72℃.			

5. Place the PCR tubes or PCR plates in the thermal cycle and start the cycling program.

Troubleshooting Guide

No PCR product or unexpected PCR products (problems occurring during reverse transcription)
1. Inappropriate cell numbers used
Try different numbers of cells per well/tube. Carry out the AmpEasy™ Cells qRT-PCR Kit (For Probe System w/ ROX) procedure and determine which cell number gives optimal PCR results. Please be cautious that RNase in the sample might not be completely inactivated when high numbers of cells are lysed.
2. Cells not washed with Buffer C or Cells treated with incorrect volume of Buffer C
Cell lysate may contain inhibitors of RT-PCR. Be sure to remove intracellular and extracellular contaminants by using Buffer C. When human cultured cells are analyzed, the optimal cell concentration is $5x10^4$ cells. 50 µl of Buffer C is required for $5x10^4$ cells.
3. Incorrect setup of reverse transcription reaction
Be sure to set up the reaction on ice.
4. High volume of reverse transcription reaction is added to real-time PCR
To prevent the reduction amplification efficiency and the linearity of the reaction, the volume of reverse transcription reaction added should not exceed 10% of the final PCR volume to prevent the reduction in PCR efficiency.
5. Inappropriate temperature of reverse transcription reaction
Reverse transcription should be carried out at 42℃ . The temperature range of 42-50℃ is acceptable if different temperature is required. Temperatures over 50℃ is not recommended as it might reduce the activity of RealScript™ Reverse Transcriptase and therefore affect the cDNA yield.
6. RNA denaturation
Denaturation of the template RNA is not necessary. If denaturation was performed, the integrity of the RNA may be affected.
No PCR product or unexpected PCR products (problems occurring during PCR
1. Pipetting error or missing reagent
Check the concentrations and storage conditions of the reagents, including probe, primers and template. Repeat the PCR.
2. Primer design not optimal
Check for PCR products by gel electrophoresis. If no specific PCR products are detected, review the primer design guidelines.
3. Problems with starting template
Check the concentration, storage conditions, and quality of the starting template. If necessary, make new serial dilutions of template nucleic acids from the stock solutions. Repeat the PCR using the new dilutions.