Cat. # 3736S

For Research Use

TaKaRa

CellAmp™ Direct Probe RT-qPCR Kit

Product Manual

v201809Da

Table of Contents

I. Description

CellAmp Direct Probe RT-qPCR Kit is designed for directly performing probe-based real-time RT-qPCR, without conducting RNA extraction, from various mammalian-cultured cells (adherent cells, non-adherent cells, primary cells, stem cells, iPS cells, etc.). The kit allows for the preparation of cell lysates from cultured cells and completion of real-time RT-qPCR in as little as 1.5 hours. In addition, this product works well for analysis in cases using template contaminated with genomic DNA by effectively eliminating the genomic DNA. A two-step real-time RT-PCR reagent*1 for probe detection is included in the kit, which makes the gene expression analysis easy to perform.

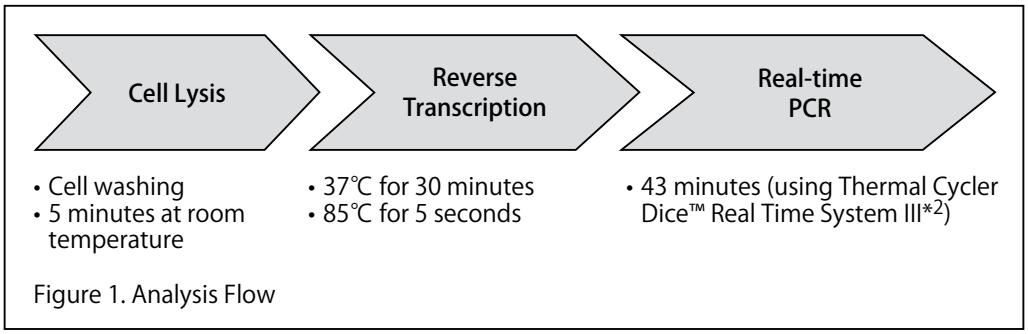


Figure 1. Analysis Flow

* 1 The qPCR reagent, Probe qPCR Mix (2X) included in this kit is equivalent to the Probe qPCR Mix (Cat. #RR391A/B).

* 2 Not available in all geographic locations. Check for availability in your area.

II. Features

1. High-throughput qPCR analysis can be performed easily and rapidly (~1.5 hours):
   Real-time PCR template is prepared directly from cells without RNA purification. This kit does not require heat treatment to inactivate DNase before reverse transcription reactions, unlike the CellAmp Direct RNA Prep Kit for RT-PCR (Real Time) (Cat. #3732). The kit is suitable for high-throughput analyses because of its simple procedure.

2. Useful for cells differentiated from stem cells and iPS cells:
   The improved CellAmp Lysis Buffer II allows this product to be used with various cultured cells: adherent cells, non-adherent cells, primary cells, stem cells, iPS cells, etc.*

   * An example of an experiment using iPS cell-derived cardiomyocytes is shown in Section VII. Experimental Example: Gene Expression Profiling.

3. The prepared cell lysates can be stored for a long time:
   This lysate is stable at -20℃ for ~ 6 months.

4. Resistance to PCR inhibitors and high specificity:
   The kit shows high-reactivity, even in the presence of PCR inhibitors and target DNA having high GC content, since Probe qPCR Mix (Cat. #RR391A/B) is used as the real-time PCR reagent.

III. Components

1.	CellAmp Washing Buffer*1, 6	2.5 ml
2.	CellAmp Lysis Buffer II*1, 6	1 ml
3.	DNase I for Direct RNA Prep*1, 6	40 µl
4.	Stop Solution*1, 6	50 µl
5.	PrimeScript™ RT Enzyme Mix*2, 6	40 µl
6.	5X CellAmp Buffer II*2, 6	80 µl
7.	RT Primer Mix*2, 3, 6	20 µl
8.	RNase-Free H_2O*2, 6	1 ml
9.	Probe qPCR Mix (2X)*4, 5	625 µl x 2
10.	ROX Reference Dye (50X)*4, 5, 7	50 µl
11.	ROX Reference Dye II (50X)*4, 5, 7	50 µl

* 1 Cell lysis reagents for 50 reactions.

* 2 RT (reverse transcription) reagent for 20 reactions.

* 3 Contains Oligo dT Primer and Random 6 mers.

* 4 qPCR reagent for 500 reactions.

* 5 Equivalent to Probe qPCR Mix (also sold separately as Cat. #RR391A/B).

* 6 Also sold in CellAmp Direct Lysis and RT set (Cat. #3737S/A) separately.

* 7 ROX Reference Dyes are used for analyses performed with instruments that normalizes fluorescent signals between wells.

◆ Add ROX Reference Dye (50X) when using the following instruments (the final concentration should be 1X):
- Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific)
- StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)

◆ Add ROX Reference Dye II (50X) when using the following instruments(the final concentration should be 0.5X):
- Applied Biosystems 7500/7500 Fast Real-Time PCR System (Thermo Fisher Scientific)

◆ No ROX Reference Dye is required when using the following instruments:
- Thermal Cycler Dice Real Time System III (Cat. #TP950/TP970/TP980/TP990)*8
- Thermal Cycler Dice Real Time System *II* (Cat. #TP900/TP960)*8
- Thermal Cycler Dice Real Time System *Lite* (Cat. #TP700/TP760)*8
- LightCycler 96/LightCycler 480 System (Roche Diagnostics)
- CFX96 Real-Time PCR Detection System (Bio-Rad)
- Smart Cycler System/Smart Cycler II System (Cepheid)

* 8 Not available in all geographic locations. Check for availability in your area.

IV. Storage

-20℃

CellAmp Washing Buffer and CellAmp Lysis Buffer II

Can be stored at 4℃ after thawing. Be sure to avoid contamination.

Probe qPCR Mix (2X)

Stable for 6 months when stored at 4℃. Be sure to avoid contamination.

- For long-term storage, store at -20℃. Once thawed, Probe qPCR Mix (2X) must be stored at 4℃ and used within 6 months.
- When using for the first time, gently mix by inverting until completely thawed and evenly mixed and then centrifuge briefly.

V. Precautions

- Perform cell lysate preparation quickly without interruption.
- Be sure to use new disposable tips to minimize the risk of contamination between samples when dispensing reagents.

<General precautions for handling RNA>

- It is likely that commercially available sterilized disposable plasticware is RNase-free and can be used for such experiments. However, microcentrifuge tubes, micropipette tips, etc. must be autoclaved before use.
- When using glassware or spatulas, perform dry-heat sterilization at 160℃ for at least 2 hours. Anything that cannot be dry-heat sterilized this way must be treated with 0.1% diethylpyrocarbonate (DEPC) at 37℃ for 12 hours, and then autoclaved (to prevent RNA carboxymethylation caused by DEPC) before use. It is important to keep equipment for RNA experiments separate from other types of equipment.
- The largest cause of RNase contamination is handling with bare hands. When an experiment is performed using RNA, disposable plastic gloves and masks must be used.

VI. Protocol

VI-1. Preparation of Lysis Solution

Prepare the following lysis solution in a microcentrifuge tube on ice.

<Per well of 96-well plate>

Reagents	Amount
CellAmp Lysis Buffer II	48 μl
DNase I for Direct RNA Prep	2 μl
Total	50 μl

VI-2. Lysate Preparation

[Adherent cells cultured in 96-well plates*1]

1. Remove as much culture medium as possible.
2. Add 125 μl/well of CellAmp Washing Buffer for washing.
3. Remove CellAmp Washing Buffer as much as possible.
4. Add 50 μl of the lysis solution, from Step VI-1, in each well and incubate for 5 minutes at room temperature (around 25℃).
5. After incubation, add 2.5 μl of Stop Solution and repeat pipetting 5 times.*2 Use the resulting mixture as the cell lysate for the protocol in VI-3.

* 1 The standard cell number is 1 x 10^4 cells/well. However, the kit works well for a large range (1 x 10^2 cells/well and 1 x 10^6 cells/well). The volume of the lysis solution remains the same regardless of the cell number.

* 2 Add 2.5 μl of Stop Solution to 50 μl of lysate. If the volume of the lysate solution is scaled up, the volume of Stop Solution must be adjusted accordingly.

Example: Add 5 μl of Stop Solution to 100 μl of lysate solution.

[Cell pellet]

Perform this method when using non-adherent cells or residual cells from subculture.

1. Transfer cells to a microcentrifuge tube.
2. Centrifuge at a suitable speed for each cell type.*3
3. Remove as much medium as possible.
4. Add 125 μl of CellAmp Washing Buffer for washing.
5. Centrifuge at a suitable speed for each cell type.*3
6. Remove as much CellAmp Washing Buffer as possible.
7. Add 50 μl of the lysis solution and incubate for 5 minutes at room temperature (around 25℃).
8. Add 2.5 μl of Stop Solution and repeat pipetting 5 times.
   Use the resulting mixture as the cell lysate in VI-3.

* 3 Since the centrifugation condition depends on the cell type, centrifuge at a speed that is suitable for the cell type.
Example: HeLa cells - 1,500 rpm for 5 minutes.

Note 1 : Cell lysates from cultured cell lines are stable for ~ 2 hours on ice.
Note 2 : For long term storage, store cell lysates at -20℃ for ~ 6 months.

VI-3. Reverse Transcription Reaction

1. Prepare a reaction master mix of the following reagents, except for cell lysate, on ice and dispense 18 μl of the mixture into each PCR tube or well of 96-well PCR plate.

<Per reaction>

Reagents	Amount
5X CellAmp Buffer II	4 μl
PrimeScript RT Enzyme Mix	1 μl
RT Primer Mix	1 μl
RNase-Free H_2O	12 μl
Total	18 μl*4

* 4 The reverse transcription reaction can be scaled up as necessary.

2. Add 2 μl*5 of cell lysate from step VI-2 to each PCR tube or well of 96-well plate and keep on ice.

* 5 The amount of cell lysate should be 1/10 or less of the solution mixture.

3. Perform the reaction under the following condition.

37℃ 30 minutes (reverse transcription)
85℃ 5 seconds (inactivation of reverse transcriptase)
4℃

VI-4. Real-Time PCR

[Thermal Cycler Dice Real Time System III, *II*, *Lite*, and other qPCR instruments*1 that are not normalized with ROX Reference Dye]

* 1 For LightCycler 96/480 System, CFX96 Real-Time PCR Detection System, or Smart Cycler System/Smart Cycler II System, also refer to the manual for Probe qPCR Mix (Cat. #RR391A/B).

1. Prepare the PCR mixture as shown below.

<Per 1 reaction>

Reagents	Amount	Final conc.
Probe qPCR Mix (2X)	12.5 μl	1 X
PCR forward primer (10 μM)	0.5 μl	0.2 μM*2
PCR reverse primer (10 μM)	0.5 μl	0.2 μM*2
Probe*3	1 μl	
RT reaction mixture*4	4 μl	
Sterile purified water	6.5 μl	
Total	25 μl*5	

* 2 The final concentration of primers can be 0.2 μM for most reactions. When that does not work, determine the optimal concentration within the range of 0.1 - 1.0 μM.

* 3 The probe concentration depends on the type of PCR instrument and fluorescently-labeled probe substance that are used. Consult the production manual of the instrument and the attached probe datasheet to determine the volume to be added. Typically, the final concentration will fall within the range of 0.1 to 0.5 μM when using the Thermal Cycler Dice Real Time System.

* 4 The volume of the RT reaction mixture should not exceed 16% of the total PCR reaction volume.

* 5 The recommended reaction volume is 25 μl for Thermal Cycler Dice Real Time System.

2. Start the reaction.

The shuttle PCR protocol is recommended for the PCR reaction. The annealing/elongation (60℃) time can vary from 20 to 30 seconds.

Shuttle PCR Protocol

Hold (initial denaturation)
Cycle : 1
95℃ 30 seconds
2-step PCR
Cycles : 40
95℃ 5 seconds
60℃ 30 seconds

* Precaution
The DNA polymerase used in this product is a hot start PCR enzyme that utilizes an anti-*Taq* antibody that inhibits polymerase activity. Do not perform the pre-PCR 5-15 minute activation step at 95℃, which is required by chemically-modified hot start PCR enzymes available from other companies. Longer heat treatment may decrease enzyme activity and affect amplification efficiency and quantification accuracy. Even for initial template denaturation before PCR, 95℃ for 30 seconds is normally sufficient.

3. After the reaction is complete, check the amplification curve.

For analysis methods, see the product manual of the real-time PCR instrument.

[Applied Biosystems 7300 Real-Time PCR System, StepOnePlus Real-Time PCR System, and other qPCR instruments*1 that require normalization with ROX Reference Dye]

* 1 Follow the instructions in the manual for each instrument.

1. Prepare the PCR mixture as shown below.

<Per reaction>

Reagents	Amount	Final conc.
Probe qPCR Mix (2X)	12.5 μl	1 X
PCR forward primer (10 μM)	0.5 μl	0.2 μM*2
PCR Reverse Primer (10 μM)	0.5 μl	0.2 μM*2
Probe*3	1 μl	
ROX Reference Dye (50X)*4	0.5 μl	1 X
RT reaction mixture*5	4 μl	
Sterile purified water	6 μl	
Total	25 μl	

* 2 The final concentration of primers can be 0.2 μM for most reactions. When that does not work, determine the optimal concentration within the range of 0.1 - 1.0 μM.

* 3 The probe concentration depends on the type of PCR instrument and fluorescently-labeled probe substance that are used. Consult the production manual of the instrument and the attached probe datasheet to determine the volume to be added.

* 4 The final concentration for ROX Reference Dye (50X) should be 1X.

* 5 The volume of the RT reaction mixture should not exceed 16% of the total real-time PCR reaction volume.

2. Start the reaction.

The shuttle PCR protocol is recommended for the PCR reaction. Try this protocol first and optimize the PCR conditions as necessary.

<7300 Real-Time PCR System>

Shuttle PCR Standard Protocol

Stage 1 : Initial denaturation
Reps : 1
95℃ 30 seconds

Stage 2 : PCR reaction
Reps : 40
95℃ 5 seconds
60℃ 31 seconds

<StepOnePlus Real-Time PCR System>

Fast Protocol

Holding Stage
Reps : 1
95℃ 20 seconds

Cycling Stage
Number of Cycles : 40
95℃ 1 second
60℃ 20 seconds

* Precaution
The DNA polymerase used in this product is a hot start PCR enzyme that utilizes an anti-*Taq* antibody that inhibits polymerase activity. Do not perform the pre-PCR 5-15 minute activation step at 95℃, which is required by chemically-modified hot start PCR enzymes available from other companies. Longer heat treatment may decrease enzyme activity and affect amplification efficiency and quantification accuracy. Even for initial template denaturation before PCR, 95℃ for 30 seconds is normally sufficient.

3. After the reaction is complete, check the amplification curve.

For analysis methods, see the product manual of the real-time PCR instrument.

[Applied Biosystems 7500/7500 Fast Real-Time PCR System and other qPCR instruments*1 that require normalization with ROX Reference Dye II]

* 1 Follow the instructions in the manual for each instrument.

1. Prepare the PCR mixture as shown below.

<Per reaction>

Reagents	Amount	Final conc.
Probe qPCR Mix (2X)	12.5 μl	1 X
PCR forward primer (10 μM)	0.5 μl	0.2 μM*2
PCR reverse primer (10 μM)	0.5 μl	0.2 μM*2
Probe*3	1 μl	
ROX Reference Dye II (50X)*4	0.25 μl	0.5X
RT reaction mixture*5	4 μl	
Sterile purified water	6.25 μl	
Total	25 μl	

* 2 The final concentration of primers can be 0.2 μM for most reactions. When that does not work, determine the optimal concentration within the range of 0.1 - 1.0 μM.

* 3 The probe concentration depends on the type of PCR instrument and fluorescently-labeled probe substance that are used. Consult the production manual of the instrument and the attached probe datasheet to determine the volume to be added.

* 4 The final concentration for ROX Reference Dye II (50X) should be 0.5X.

* 5 The volume of the RT reaction mixture should not exceed 16% of the total real-time PCR mixture.

2. Start the reaction.

The following shuttle PCR standard protocol is recommended for the PCR reaction. Try this protocol first and optimize the PCR conditions as necessary.

<7500 Real-Time PCR System>
Shuttle PCR Standard Protocol
Stage 1 : Initial denaturation
Reps : 1
95℃ 30 seconds
Stage 2 : PCR reaction
Reps : 40
95℃ 5 seconds
60℃ 34 seconds

<7500 Fast Real-Time PCR System>
Fast Protocol
Holding Stage
Reps : 1
95℃ 20 seconds
Cycling Stage
Number of Cycles : 40
95℃ 3 second
60℃ 30 seconds

* Precaution
The DNA polymerase used in this product is a hot start PCR enzyme that utilizes an anti-*Taq* antibody that inhibits polymerase activity. Do not perform the pre-PCR 5-15 minute activation step at 95℃, which is required by chemically-modified hot start PCR enzymes available from other companies. Longer heat treatment may decrease enzyme activity and affect amplification efficiency and quantification accuracy. Even for initial template denaturation before PCR, 95℃ for 30 seconds is normally sufficient.

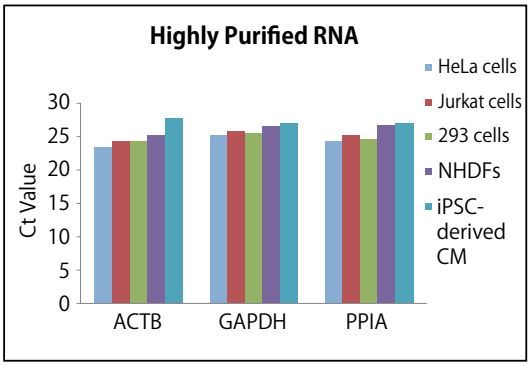
3. After the reaction is complete, check the amplification curve.

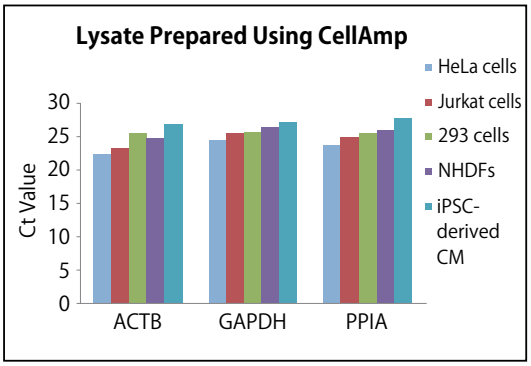
For analysis methods, see the product manual of the real-time PCR instrument.

VII. Experimental Example: Gene Expression Profiling

<Method> A total of 1 x 10^4 cells was collected from HeLa cells, Jurkat cells, 293 cells, normal human dermal fibroblasts (NHDF), and cardiomyocytes (CM) induced from iPS cells and 50 μl of cell lysate was prepared for each according to the protocol (see VI-2) for use as templates. An RT reaction and a real-time PCR were performed according to the protocol. Purified total RNA (from 1 x 10^4 cells) were also prepared as a control and subjected to the same gene expression analysis.

<Result> The gene expression profile obtained from lysate prepared with this kit is found to be similar to that of the highly purified RNA.





VIII. Troubleshooting

<No amplification observed in real-time RT-PCR>

- Check if amplification can be observed when performing a real-time RT-PCR using, total RNA that was highly purified with NucleoSpin RNA (Cat. #740955.10/.50/.250)* or RNAiso Plus (Cat. #9108/9109).
- Consider redesigning PCR primers and probes. To perform a real-time RT-PCR efficiently, it is important to design highly reactive PCR primers and probes.
- The experimental protocol must be optimized depending on the specific cell types and cultivation conditions.
- Wash cells with CellAmp Washing Buffer and remove contaminants in the culture medium. Also, remove as much of the culture medium and CellAmp Washing Buffer as possible.
- Prepare real-time PCR reaction mixture on ice. It should be kept on ice and protected from light until the reaction is started.
- If an excessive volume of lysate is added to the RT reaction in the 2-step real-time RT-PCR, the reaction efficiency may decrease.

* Not available in all geographic locations. Check for availability in your area.

IX. Related Products

[Real-time PCR instrument]
Probe qPCR Mix (Cat. #RR391A/B)

[Real-time PCR equipment]
Thermal Cycler Dice™ Real Time System III (Cat. #TP950/TP970/TP980)*1
Thermal Cycler Dice™ Real Time System *II* (Cat. #TP900/TP960)*1
Thermal Cycler Dice™ Real Time System *Lite* (Cat. #TP700/TP760)*1

[For intercalater method]
CellAmp™ Direct TB Green™ RT-qPCR Kit (Cat. #3735S/A)*2

[Additional reagents]
CellAmp™ Direct Lysis and RT set (Cat. #3737S/A)

*1 Not available in all geographic locations. Check for availability in your area.

*2 We have begun the process of changing the names for Takara Bio's intercalator-based real-time PCR (qPCR) products to the "TB Green series". These products can be used the same way as before, as only the names are changing. Catalog number and product performance are unaffected by this transition.