Cat. # 3735S

For Research Use

TaKaRa

CellAmp™ Direct TB Green™ RT-qPCR Kit

Product Manual

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.

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I. Description

CellAmp Direct TB Green RT-qPCR Kit is designed for performing a quick real-time RT-PCR by intercalater method using TB Green for detection, without the need for RNA extraction from various cultured mammalian cells (adherent cells, non-adherent cells, primary cells, stem cells, iPS cells, etc.). The kit allows for preparation of cell lysate from cultured cells for performing reverse transcription reactions and real-time PCR in as little as 1.5 hours. In addition, this kit works well in case of analyses using templates contaminated by genomic DNA by effective elimination of genomic DNA. Two-step real-time RT-PCR reagent*1 for TB Green detection is included in the kit, which makes a gene expression analysis easy to perform.

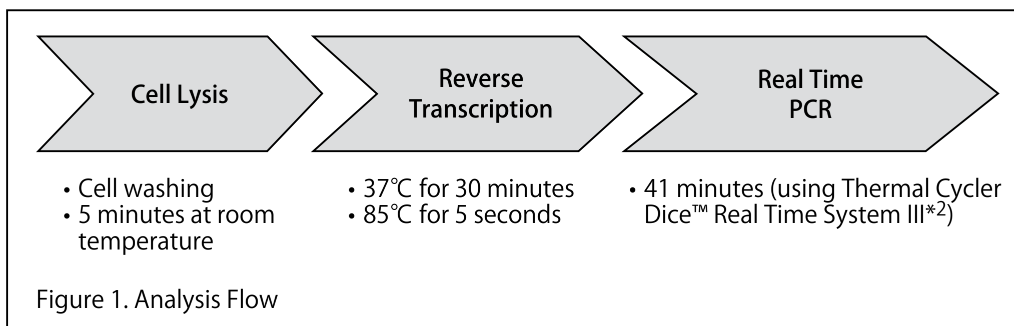


Figure 1. Analysis Flow

* 1 The qPCR reagent, TB Green Fast qPCR Mix (2X) included in this kit is equivalent to the TB Green Fast qPCR Mix (Cat. #RR430S/A/B).
* 2 Not available in all geographic locations. Check for availability in your area.

II. Features

1. High-throughput qPCR analyses 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 CellAmp Direct RNA Prep Kit for RT-PCR (Real Time) (Cat. #3732). The kit is well suited 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.*

   * 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:
   The cell lysates obtained by this kit are 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 TB Green Fast qPCR Mix (Cat. #RR430A/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.	TB Green Fast 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 reagent for 20 reactions.
* 2 RT (reverse transcription) reagent for 20 reactions.
* 3 Contains Oligo dT Primer and Random 6 mers.
* 4 qPCR reagent for 100 reactions.
* 5 Equivalent to TB Green Fast qPCR Mix (also sold seperately as Cat. #RR430S/A/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 normalize fluorescent signals between wells.

◆ Add ROX Reference Dye (50X) when using the following instruments:
- 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:
- 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.

TB Green Fast qPCR Mix (2X)

Stable for 6 months when stored at 4℃ . Protect it from light and avoid contamination.

- For long-term storage, store at -20℃. Once thawed, TB Green Fast qPCR Mix (2X) must be stored at 4℃ and used within 6 months.
- When using for the first time, gently mix by inverting until it is 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 RNA experiment equipment 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 of the culture medium as possible.
2. Add 125 μl of CellAmp Washing Buffer to each well for washing.
3. Remove as much of the CellAmp Washing Buffer 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. Add 2.5 μl of Stop Solution and repeat pipetting 5 times. Use as cell lysate in step VI-3.*2

* 1 The standard cell number is 1 x 10^4cells/well. However, the kit works well for a large range (1 x 10^2 cells to 1 x 10^6 cells). 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 the 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 Pellets]

Perform this method when using non-adherent cells or residual cells from cell 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 from step VI-1, and incubate for 5 minutes at room temperature (around 25℃).
8. After incubation, add 2.5 μl of Stop Solution and repeat pipetting 5 times. Use the resulting mixture as cell lysate in step 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

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

<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 microtube 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, refer to the manual for TB Green Fast qPCR Mix (Cat. #RR430A/B).

1. Prepare a PCR mixture as shown below.

<Per 1 reaction>

Reagents	Amount	Final conc.
TB Green Fast qPCR Mix (2X)	12.5 μl	1 X
PCR Forward Primer (10 μM)	1 μl	0.4 μM*2
PCR Reverse Primer (10 μM)	1 μl	0.4 μM*2
RT reaction mixture*3	4 μl	
Sterile purified water	6.5 μl	
Total	25 μl*4	

* 2 The final concentration of primers can be 0.4 μM in most reactions. If it does not work, determine the optimal concentrations within the range of 0.2 - 1.0 μM.
* 3 The volume of the RT reaction mixture should be no more than 16% of real-time PCR mixture.
* 4 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 PCR reaction. Use this protocol first and optimize the PCR conditions as necessary. Perform 3-step PCR when a shuttle PCR is not feasible, for example, for primers with low Tm values.

Shuttle PCR Standard Protocol

Hold (initial denaturation)
Cycle : 1
95℃ 30 seconds
2 Step PCR
Cycles : 40
95℃ 5 seconds
60℃ 10 seconds
Dissociation

* 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 a chemically-modified hot-start PCR enzyme 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 and melting curves. For analysis methods, see the product manual for the real-time PCR instrument.

[Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System, StepOnePlus Real-Time PCR System, and other qPCR instruments that are normalized with ROX Reference Dye]

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

1. Prepare a PCR mixture as shown below.

<Per reaction>

Reagents	Amount	Final conc.
TB Green Fast qPCR Mix (2X)	12.5 μl	1X
PCR Forward Primer (10 μM)	1 μl	0.4 μM*1
PCR Reverse Primer (10 μM)	1 μl	0.4 μM*1
ROX Reference Dye (50X) or Dye II (50X)*2	0.5 μl	1X
RT reaction mixture*3	4 μl	
Sterile purified water	6 μl	
Total	25 μl	

* 1 The final concentration of primers can be 0.4 μM in most reactions. If it does not work, determine the optimal concentrations within the range of 0.2 - 1.0 μM.

* 2 The concentration of ROX Reference Dye II (50X) is lower than that of ROX Reference Dye (50X). Use ROX Reference Dye II (50X) for performing analyses with Applied Biosystems 7500 Real-Time PCR System or 7500 Fast Real-Time PCR System.
Use ROX Reference Dye (50X) for performing analyses with StepOnePlus or 7300 Real-Time PCR System.

* 3 The volume of the RT reaction mixture should be no more than 16% of real-time PCR reaction volume.

2. Start the reaction.

The shuttle PCR protocol is recommended for PCR reaction. Use this protocol first and optimize the PCR conditions as necessary. Perform 3-step PCR when a shuttle PCR is not feasible, for example, for primers with low Tm values.

<Applied Biosystems 7300/7500 Real-Time PCR System, StepOnePlus>

Shuttle PCR Standard Protocol

Stage 1 : Initial denaturation
Reps : 1
95℃ 30 seconds
Stage 2 : PCR reaction
Reps : 40
95℃ 5 seconds
60℃ 10 seconds ~ 15 seconds*4
Stage 3 : Melt Curve

* 4 Select a filter to use (FAM, ROX), and set the shortest length of time that allows detection by the filter. The length can be set at 10 seconds for StepOnePlus.

< Applied Biosystems 7500 Fast Real-Time PCR System>

Shuttle PCR Standard Protocol

Holding Stage
Reps : 1
95℃ 30 seconds
Cycling Stage
Number of Cycles : 40
95℃ 3 seconds
60℃ 12 seconds ~ 15 seconds*5
Melt Curve Stage

* 5 Select a filter to use (FAM, ROX), and set the shortest length of time that allows detection by the filter.

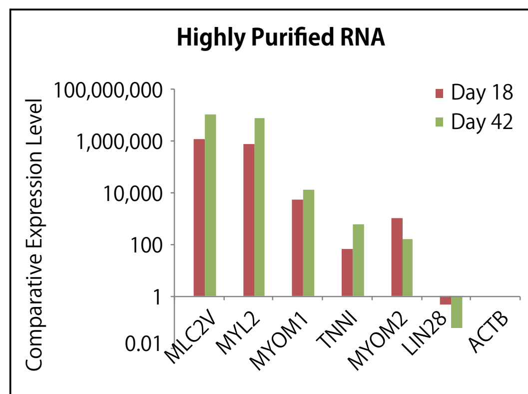
> * Precaution
> The DNA polymerase used in this product is a hot start PCR enzyme that utilizes an anti-*Taq* antibody that inhibits polymerase activity. Do not perform the pre-PCR (5–15 minute activation step) at 95℃ which is required by a chemically-modified hot-start PCR enzyme available from other 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.

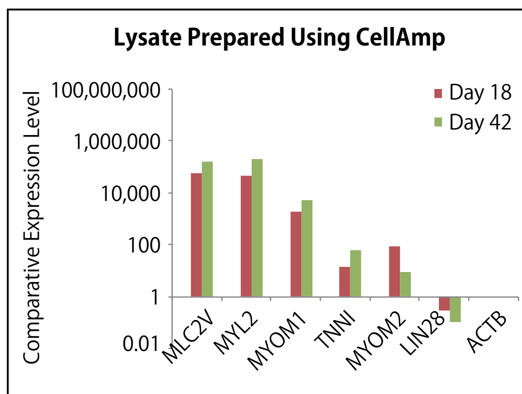
3. After the reaction is complete, check the amplification and melting curves.
   For analysis methods, see the manual for the real-time PCR instrument.

VII. Experimental Example: Gene Expression Profiling

<Method> Cardiomyocyte differentiation of iPS cells was induced. At 5 days, 18 days, and 42 days after the induction, 1 x 10^4 cells were sampled and each 50 μl of cell lysate from 1 x 10^4 cells was prepared according to the protocol. An RT reaction and a real-time PCR were performed according to the protocol, and a gene expression analysis was conducted. Purified total RNA extracted from 1 x 10^4 cells was prepared as the control, and was subjected to the same gene expression analysis.

<Result> The gene expression levels at Day 18 and 42 were compared with that at Day 5 as 1. The gene expression profile obtained from the lysate prepared with the kit was 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 highly purified with NucleoSpin RNA (Cat. #740955.10/.50/.250)* or RNAiso Plus (Cat. #9108/9109) as control.
- Consider re-designing PCR primers. To perform a real-time RT-PCR efficiently, it is important to design highly reactive PCR primers.
- The experimental protocol must be optimized depending on the cell types and cultivation condition.
- 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 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 reagent]
TB Green™ Fast qPCR Mix (Cat. #RR430A/B)

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

[For probe detection]
CellAmp™ Direct Probe RT-qPCR Kit (Cat. #3736S/A)*

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

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

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