Cat. # 3732

For Research Use

TaKaRa

CellAmp™ Direct RNA Prep Kit for RT-PCR (Real Time)

Product Manual

v202201Da

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I. Description

The CellAmp Direct RNA Prep Kit for RT-PCR (Real Time) is for preparation of templates used in One-step and Two-step real time RT-PCR (also called qRT-PCR or RT-qPCR). This simple, 3 solution kit can be used to prepare templates for real time RT-PCR from cultured cells (96-well plates or other sizes) in approximately 10 minutes. Using this kit with a one-step real time RT-PCR kit (e.g. One Step TB Green® PrimeScript™ RT-PCR Kit (Perfect Real Time) (Cat #RR066A/B) allows gene expression analysis to be performed in approximately 2 hours. This kit may also be used to prepare template cDNA for real time PCR (qPCR) in about 30 minutes when used in combination with a kit for reverse transcription such as PrimeScript RT reagent Kit (Perfect Real Time) (Cat #RR037A/B).
Templates can be prepared directly from small quantities of cells which is useful for gene expression profile analysis and does not influence the high sensitivity of real time RT-PCR. Genomic DNA can be removed efficiently using this kit. Therefore, it is powerful for gene expression analysis when primers can't be designed on both ends of an exon junction, or when expression levels are low.

II. Components (200 reactions)*

(1) CellAmp Washing Buffer	12.5 ml x 2
(2) CellAmp Processing Buffer	10 ml
(3) DNase I for Direct RNA Prep	200 μl

* For 200 wells of cultured cells in 96-well plate

III. Storage

-20℃

Note: CellAmp Washing Buffer and CellAmp Processing Buffer can be stored at 4℃ after thawing. Avoid contamination.

IV. Materials Required but not Provided

Reagents

- Real time RT-PCR kit (Table1)
- Proteinase K (Cat. #9034)
  For optional Proteinase K protocol (Section VII.4).

V. General Considerations

1. This kit may be used in combination with the products listed in Table 1.

Table 1. Compatible real time RT-PCR products

One-step real time RT-PCR

Cat. #	Product
RR066A/B	One Step TB Green PrimeScript RT-PCR Kit (Perfect Real Time)*
RR086A/B	One Step TB Green PrimeScript RT-PCR Kit II (Perfect Real Time)
RR096A/B	One Step TB Green PrimeScript PLUS RT-PCR Kit (Perfect Real Time)*
RR064A/B	One Step PrimeScript RT-PCR Kit (Perfect Real Time)

Two-step real time RT-PCR

Cat. #	Product
RR037A/B	PrimeScript RT reagent Kit (Perfect Real Time)
RR036A/B	PrimeScript RT Master Mix (Perfect Real Time)
RR820A/B	TB Green *Premix Ex Taq*™ II (Tli RNaseH Plus)
RR420A/B	TB Green *Premix Ex Taq* (Tli RNaseH Plus)
RR091A/B	TB Green Premix DimerEraser™ (Perfect Real Time)*
RR390A/B	*Premix Ex Taq* (Probe qPCR)

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

2. If precipitate appears during thawing of CellAmp Washing Buffer and CellAmp Processing Buffer, dissolve precipitate completely before using by warming up to room temperature.
3. Perform the lysate preparation quickly.
4. Be careful to prevent contamination between samples by using new disposable tips for dispensing the reagents. If additional reagent must be withdrawn, change the tip first. Do not use the same tip to pipette different reagents.

5. Guidelines for RNA preparation
    a. Sterilized disposable RNase-free plasticware should be used for these experiments. Any plasticware that is not certified RNase-free should be autoclaved before use. When using glass equipment or spatula, perform dry heat sterilization at 160℃ for at least 2 hours. If dry heat sterilization cannot be performed, treat with 0.1% Diethylpyrocarbonate (DEPC) at 37℃ for 12 hours, then autoclave.
    b. It is important to use separate equipment dedicated to RNA experiments.
    c. Reagents should be prepared with 0.1% DEPC treated water as much as possible, and autoclave before use. If reagents are to be used which cannot be autoclaved, then use sterilized equipment and water to prepare the solution, and then perform filter sterilization before use.
    d. Extra precautions should be taken during sample preparation, including use of clean disposable gloves and avoiding RNase contamination from operator sweat or saliva during assembly.

VI. Protocol

1. Preparation of reagents

Prepare the Processing Solution in a microcentrifuge tube on ice.

Reagent	Per well (96-well plate)
CellAmp Processing Buffer	49 μl
DNase I for Direct RNA Prep	1 μl
Total	50 μl

Note: Please refer to Section VII.3 if other types of plates are to be used.

2. Preparation of cell lysate from adherent cells cultured on 96-well plates

Note: If a different plate is used, please refer to Section VII.3 for recommended cells/well and reagent volumes.

a. Dispense about 1 x 10^4 cells in each well of 96-well plate.
b. Incubate until cells reach appropriate numbers or confluency.
c. Remove as much culture medium as possible.
d. Add 125 μl of CellAmp Washing Buffer to each well and then remove as much Washing Buffer as possible.
e. Add 50 μl of the Processing Solution to each well, then incubate for 5 minutes at room temperature (15 – 28℃).
f. Pipette cell lysate in each well several times and then transfer the lysate to a PCR tube or microcentrifuge tube. Incubate for 5 minutes at 75℃.
g. Perform real time RT-PCR using the prepared lysate as a template.
    - Follow the protocol described in Section VII.1 (one-step) or Section VII.2 (two-step).
    - For a 25 μl reaction volume of one-step real time RT-PCR or 10 μl of reverse transcription, use less than 2 μl of the lysate.
    - The prepared lysate should be kept on ice and real time RT-PCR should start within 1 hour of lysate preparation. The lysate can be also stored for about 2 weeks at −80℃.

3. Preparation of cell lysate from less than 1 x 10^4 non-adherent cells

Note: If preparing a lysate from > 1 x 10^4 cells, use more reagents (proportionally).

a. Count cells and transfer less than 1 x 10^4 cells to a microcentrifuge tube.
b. Centrifuge at 300*g* for 5 minutes.
c. Remove as much culture medium as possible.
d. Add 125 µl of CellAmp Washing Buffer.
e. Centrifuge at 300*g* for 5 minutes.
f. Remove as much CellAmp Washing Buffer as possible.
g. Add 50 µl of the Processing Solution and incubate for 5 minutes at room temperature (15 – 28℃).
h. Pipette cell lysate several times and then transfer the lysate to a PCR tube or microcentrifuge tube. Incubate for 5 minutes at 75℃.
i. Perform real time RT-PCR using the prepared lysate as a template
   - Follow the protocol described in Section VII.1 (one-step) or Section VII.2 (two-step).
   - For a 25 µl reaction volume of one-step real time RT-PCR or 10 µl of reverse transcription, use less than 2 µl of the lysate.
   - The prepared lysate should be kept on ice and real time RT-PCR should start within 1 hour of lysate preparation. The lysate can be also stored for about 2 weeks at −80℃.

VII. Appendix

1. Experimental example of one-step real time RT-PCR using One Step TB Green PrimeScript RT-PCR Kit II (Perfect Real Time) with Thermal Cycler Dice™ Real Time System // (discontinued)

a. Add 1 – 2 μl of cell lysate to a PCR reaction tube or 96-well plate and then place the tube/plate on ice.

b. Prepare master mixture on ice.

- Volume recommendations are per reaction.
- The final concentration of primers can be 0.4 μM in most reactions. When it does not work, determine the optimal concentrations within the range of 0.2 – 1.0 μM.

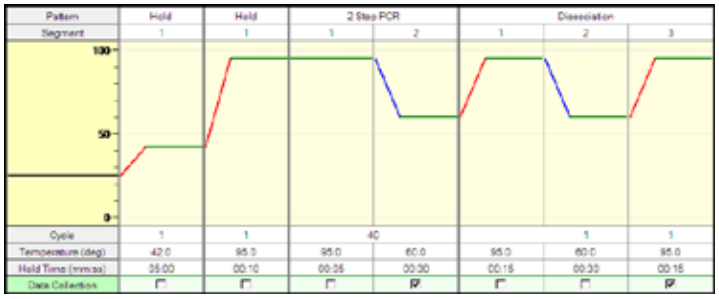
Reagent	Volume	Final conc.
2X One Step TB Green RT-PCR Buffer 4	12.5 μl	1X
PrimeScript 1 step Enzyme Mix 2	1.0 μl	
PCR Forward Primer (10 μM)	1.0 μl	0.4 μM
PCR Reverse Primer (10 μM)	1.0 μl	0.4 μM
RNase Free dH_2O	7.5 – 8.5 μl	
Total	23 – 24 μl	

c. Start reaction

- Add master mix to the cell lysate in PCR tube or 96-well plate and mix well.
- Centrifuge the PCR tube or plate briefly, set on Thermal Cycler Dice Real Time System // (discontinued) and start reaction.

Note:

When using PrimeScript 1 step Enzyme Mix 2, the initial denaturation step prior to PCR should be at 95℃ for 10 sec. Enzyme activity decreases with longer heat treatments and the amplification efficiency and quantification accuracy can be affected.



Pattern 1 : Reverse Transcription
Hold
42℃ 5 min
95℃ 10 sec
Pattern 2 : PCR
Cycles : 40
95℃ 5 sec
60℃ 30 sec
Pattern 3 : Dissociation

d. Reaction analysis

After completing reaction, verify amplification curve and dissociation curve.

2. Experimental example of two-step real time RT-PCR using PrimeScript RT reagent Kit (Perfect Real Time) and TB Green *Premix Ex Taq* II (Tli RNaseH Plus) with Thermal Cycler Dice Real Time System *II* (discontinued)

a. Reverse transcription : PrimeScript RT reagent Kit (Perfect Real Time)

1) Prepare the reaction mixture on ice.

- Volume recommendations are per reaction.
- Prepare a slightly larger amount of master mixture than is required for your number of reactions to compensate for pipetting losses. After dispensing aliquots of this mixture into the microtubes, add the RNA sample.

Reagent	Volume	Final conc.
5X PrimeScript Buffer (for Real Time)	2 μl	1X
PrimeScript RT Enzyme Mix I	0.5 μl	
Oligo dT Primer (50 μM)	0.5 μl	25 pmol
Random 6 mers (100 μM)	0.5 μl	50 pmol
RNase Free dH_2O	4.5 – 5.5 μl	
Total	8 – 9 μl	

Note:

- Efficient synthesis of cDNA from total RNA can be accomplished using both Oligo dT Primer and Random 6 mers.
- It is possible to scale up the RT reaction as needed.

Primer	Volume	Total Amount (pmol)
Oligo dT Primer (50 μM)	0.5 μl	25 pmol
Random 6 mers (100 μM)	0.5 μl	50 pmol
Gene specific primer (2 μM)	0.5 μl	1 pmol

2) Add the master mixture prepared above to a reaction tube, and then add 1 – 2 μl of the cell lysate and mix well. Centrifuge the tube briefly, and then incubate it using the following conditions :

37℃ 15 min (Reverse transcription)
85℃ 5 sec (Inactivation of reverse transcriptase with heat treatment)
4℃

Note:

- Up to 2 μl of the cell lysate can be reverse transcribed in 10 μl of the reaction mixture.
- When using a gene specific primer, perform the reverse transcription at 42℃ for 15 minutes. If non-specific amplification products are observed at the PCR step, resetting this temperature to 50℃ may improve the results.
- When the reaction mixture obtained in step 2 is used for real time PCR, the volume of the mixture should be less than 10% of the total real time PCR reaction volume.

b. Real time PCR : TB Green *Premix Ex Taq* II (Tli RNaseH Plus)

1) Add 2 μl of the reverse transcription reaction mixture to a PCR reaction tube or 96-well plate and then place on ice.

2) Prepare master mixture on ice.

- Volume recommendations are per reaction.
- The final concentration of primers can be 0.4 μM in most reactions. When it does not work, determine the optimal concentrations within the range of 0.2 – 1.0 μM.

Reagent	Volume	Final conc.
TB Green *Premix Ex Taq* II (2X)	12.5 μl	1X
PCR Forward Primer (10 μM)	1.0 μl	0.4 μM
PCR Reverse Primer (10 μM)	1.0 μl	0.4 μM
Sterile purified water	8.5 μl	
Total	23 μl	

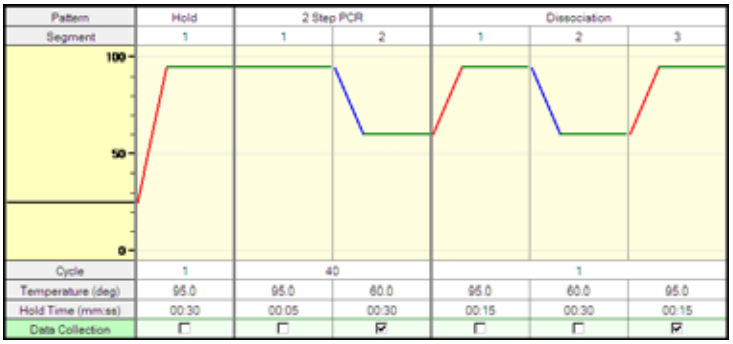
3) Start Reaction

- Add master mix to the cell lysate in PCR tube or 96-well plate and mix well.
- Centrifuge the PCR tube or plate briefly, set on Thermal Cycler Dice Real Time System *II* (discontinued) and start reaction.

Note:

- The recommended PCR conditions (Shuttle PCR standard protocol) are below. Try this protocol first and then change the PCR reaction conditions as needed. 3 Step PCR often works better with primers that have lower T_m values.
- When using TB Green *Premix Ex Taq* II, the initial denaturation step prior to PCR should be at 95℃ for 30 sec. Enzyme activity decreases with longer heat treatments and the amplification efficiency and quantification accuracy can be affected.

[Shuttle PCR standard protocol]



Hold (denaturing)
Cycle : 1
95℃ 30 sec
2 step PCR
Cycles : 40
95℃ 5 sec
60℃ 30 sec
Dissociation

4) Reaction Analysis

After completing reaction, verify amplification curve and dissociation curve, and create standard curve if quantitative analysis is necessary.

3. Adherent cell number and reagent volume per well of culture plate used

	96-well	48-well	24-well	12-well	6-well
Dispensed cells/well	1 x 10^4	2 x 10^4	4 x 10^4	8 x 10^4	2 x 10^5
CellAmp Washing Buffer	125 µl	250 µl	500 µl	1 ml	2.5 ml
CellAmp Processing Buffer	49 µl	98 µl	196 µl	392 µl	980 µl
DNase I for Direct RNA Prep	1 µl	2 µl	4 µl	8 µl	20 µl

Note: These recommendations are for commonly used adherent cells and culture conditions. The experimental protocol may need to be optimized for your cell number and culture conditions.

4. Proteinase K protocol (optional)

Reproducible results are obtained by preparing cell lysates following the protocol (Sections VI-1 to VI-3). However, if Ct-variance is observed when treating many samples at once, then this alternate protocol should be followed.

a. Preparation of reagents (96-well plate).

Note: If a different plate is used, please refer to Section VII.4-d.

1) Prepare the Processing Solution in a microcentrifuge tube on ice.

Reagent	Per 5-wells of 96-well plate
CellAmp Processing Buffer	199 µl
Proteinase K (Cat. #9034)	1 µl
Total	200 µl

2) Prepare DNase solution on ice.

Reagent	Per 1-well of 96-well plate
CellAmp Processing Buffer	9 µl
DNase I for Direct RNA Prep	1 µl
Total	10 µl

b. Preparation of cell lysate from adherent cells cultured on 96-well plates

Note: If a different plate is used, please refer to Section VII.4-d for recommended cells/well and reagent volumes.

1) Dispense about 1 x 10^4 cells in each well of 96-well plate.
2) Incubate until cells reach appropriate numbers or confluency.
3) Remove as much culture medium as possible.
4) Add 125 µl of CellAmp Washing Buffer to each well.
5) Remove as much CellAmp Washing Buffer as possible.
6) Add 40 µl of the Processing Solution to each well, then incubate for 5 min at room temperature (15 – 28℃) .
7) Pipette cell lysate in each well several times and then transfer the lysate to a PCR tube or microcentrifuge tube. Incubate for 5 min at 75℃.
8) Cool down on ice and add 10 µl of DNase solution to each well. Incubate for 5 minutes at 37℃ and then at 75℃ for 5 min. (If DNase treatment will not be performed, skip to step 9.)
9) Perform real time RT-PCR using the prepared lysate as a template.
   - Follow the protocol described in Section VII.1 (one-step) or Section VII.2 (two-step).
   - For a 25 µl reaction volume of one-step real time RT-PCR or 10 µl of reverse transcription, use less than 2 µl of the lysate.
   - The prepared lysate should be kept on ice and real time RT-PCR should start within 1 hour of lysate preparation. The lysate can be also stored for about 2 weeks at −80℃.

c. Preparation of cell lysate from less than 1 x 10^4 non-adherent cells

Note: If preparing a lysate from > 1 x 10^4 cells, use more reagents (proportionally).

1) Count cells and transfer less than 1 x 10^4 cells a to microcentrifuge tube.
2) Centrifuge at 300*g* for 5 min.
3) Remove as much culture medium as possible.
4) Add 125 µl of CellAmp Washing Buffer.
5) Centrifuge at 300*g* for 5 min.
6) Remove as much CellAmp Washing Buffer as possible.
7) Add 40 µl of the Processing Solution and incubate for 5 min at room temperature (15 – 28 ℃).
8) Pipette cell lysate in each well several times and then transfer the lysate to a PCR tube or microcentrifuge tube. Incubate for 5 min at 75℃.
9) Cool down on ice and add 10 µl of DNase solution to each well. Incubate for 5 min at 37℃ and then at 75℃ for 5 min. (If DNase treatment will not be performed, skip to step 10.)
10) Perform real time RT-PCR using the prepared lysate as a template.
    - Follow the protocol described in Section VII.1 (one-step) or Section VII.2 (two-step).
    - For a 25 µl reaction volume of one-step real time RT-PCR or 10 µl of reverse transcription, use less than 2 µl of the lysate.
    - The prepared lysate should be kept on ice and real time RT-PCR should start within 1 hour of lysate preparation. The lysate can be also stored for about 2 weeks at −80℃.

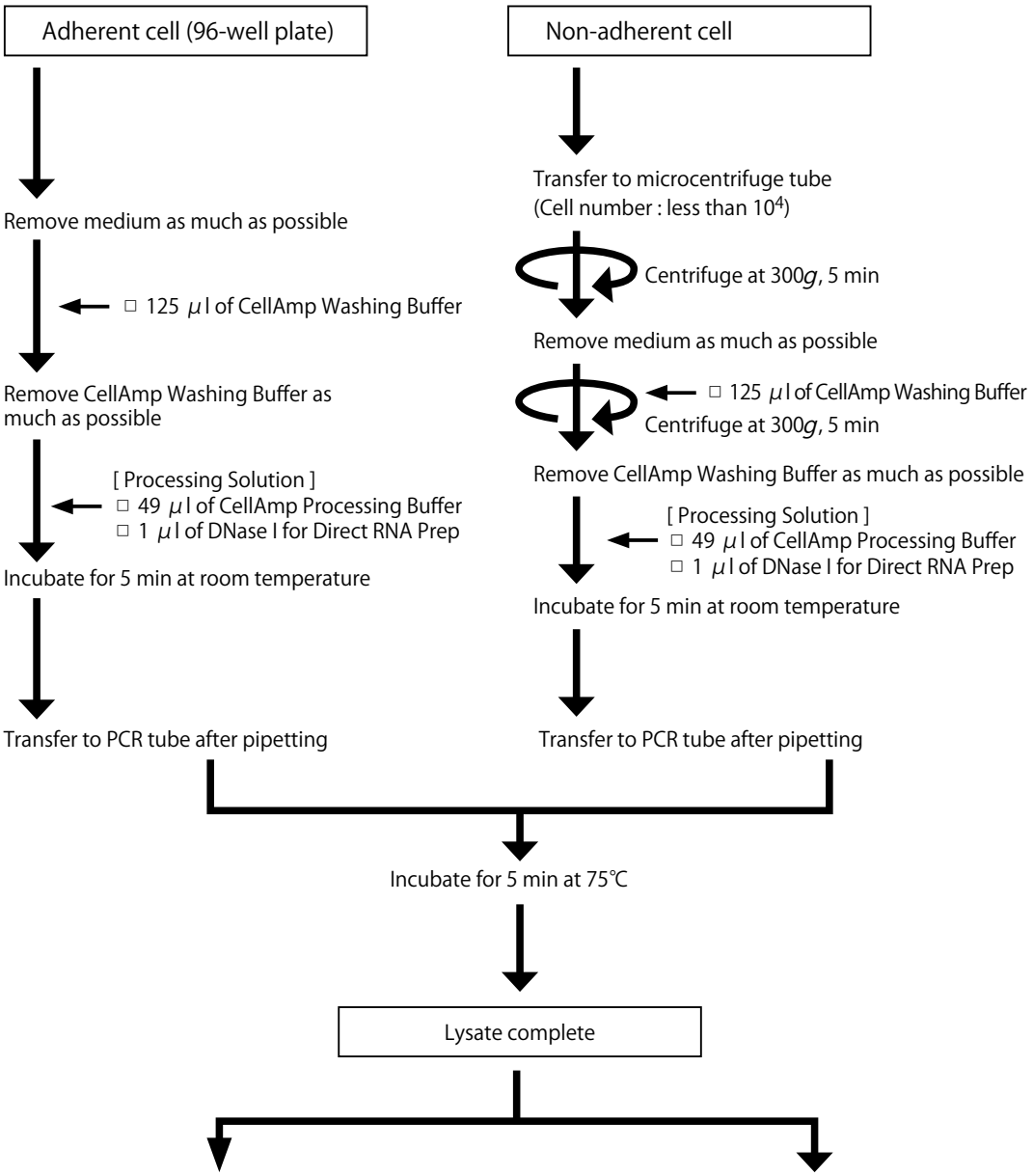
d. Adherent cell number and reagent volume per well of culture plate used

[Proteinase K protocol]

	96-well	48-well	24-well	12-well	6-well
Dispensed cells/well	1 x 10^4	2 x 10^4	4 x 10^4	8 x 10^4	2 x 10^5
CellAmp Washing Buffer	125 µl	250 µl	500 µl	1.0 ml	2.5 ml
[Processing Solution]					
CellAmp Processing Buffer	39.8 µl	79.6 µl	159.2 µl	318.4 µl	796 µl
Proteinase K (Cat. #9034)	0.2 µl	0.4 µl	0.8 µl	1.6 µl	4 µl
[DNase solution]					
CellAmp Processing Buffer	9 µl	18 µl	36 µl	72 µl	180 µl
DNase I for Direct RNA Prep	1 µl	2 µl	4 µl	8 µl	20 µl

Note: These recommendations are for commonly used adherent cells and culture conditions. The experimental protocol may need to be optimized for your cell number and culture conditions.

CellAmp Direct RNA Prep Kit Flow Chart



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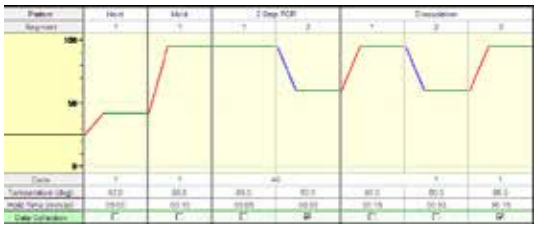
1 step real time RT-PCR

Add 1 – 2 μl of cell lysate to reaction tube or a well

One Step TB Green PrimeScript RT-PCR Kit II (Cat. #RR086A/B)
[Master Mix]
< per reaction >

□ RNase Free dH_2O	7.5 – 8.5 μl
□ 2X One Step TB Green RT-PCR Buffer 4	12.0 μl
□ PCR Forward Primer (10 μM)	1.0 μl
□ PCR Reverse Primer (10 μM)	1.0 μl
□ PrimeScript 1 step Enzyme Mix 2	1.0 μl

Start reaction



Pattern 1 : Reverse transcription
Hold
42℃ 5 min
95℃ 10 sec
Pattern 2 : PCR
Cycles : 40
95℃ 5 sec
60℃ 30 sec
Pattern 3 : Dissociation

Analyze after reaction is completed.

2 step real time RT-PCR

PrimeScript RT reagent Kit (Cat. #RR037A/B)
[Master Mix]
< per reaction >

□ RNase Free dH_2O	4.5 – 5.5 μl
□ 5X PrimeScript Buffer (for Real Time)	2.0 μl
□ Oligo dT Primer (50 μM)	0.5 μl
□ Random 6 mers (100 μM)	0.5 μl
□ PrimeScript RT Enzyme Mix I	0.5 μl

Add 1 – 2 μl of cell lysate to a reaction tube or a well

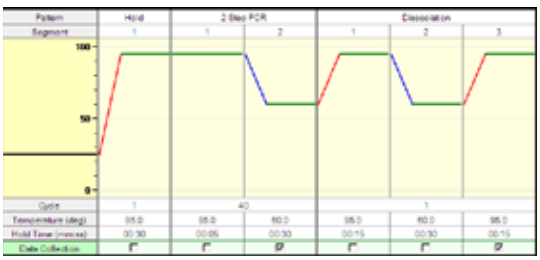
37℃ 15 min (reverse transcription)
85℃ 5 sec (Inactivation)
4℃

Add 2 μl of reaction mixture of reverse transcription to a tube or a well.

TB Green *Premix Ex Taq* II (Cat. #RR820A/B)
[Master Mix]
< per reaction >

□ Sterile purified water	8.5 μl
□ PCR Forward Primer (10 μM)	1.0 μl
□ PCR Reverse Primer (10 μM)	1.0 μl
□ TB Green *Premix Ex Taq* II (2X)	12.5 μl

Start reaction



Pattern 1 : (Initial denaturing)
Hold
95℃ 30 sec

Pattern 2 : PCR
Cycles : 40
95℃ 5 sec
60℃ 30 sec
Pattern 3 : Dissociation

Analyze after reaction is completed.

VIII. Experimental Example

Analysis of Gene expression profiling

Methods :

- HeLa cells were dispensed in a 96-well plate at 1 x 10^4, 1 x 10^3, or 1 x 10^2 cells/well. Cells were cultured for 48 hours, and then cell lysates were prepared following the protocol.
- Gene expression analysis was performed with 1 step and 2 step real time RT-PCR using 6 target genes.
- Purified total RNA (100 ng) was used as an experimental control.

Result :

- Gene expression analysis performed with the 6 genes resulted in a stable gene expression profile, which was similar to the result obtained with purely prepared RNA, was obtained from each of different cell numbers by 1 step and 2 step real time RT-PCR.

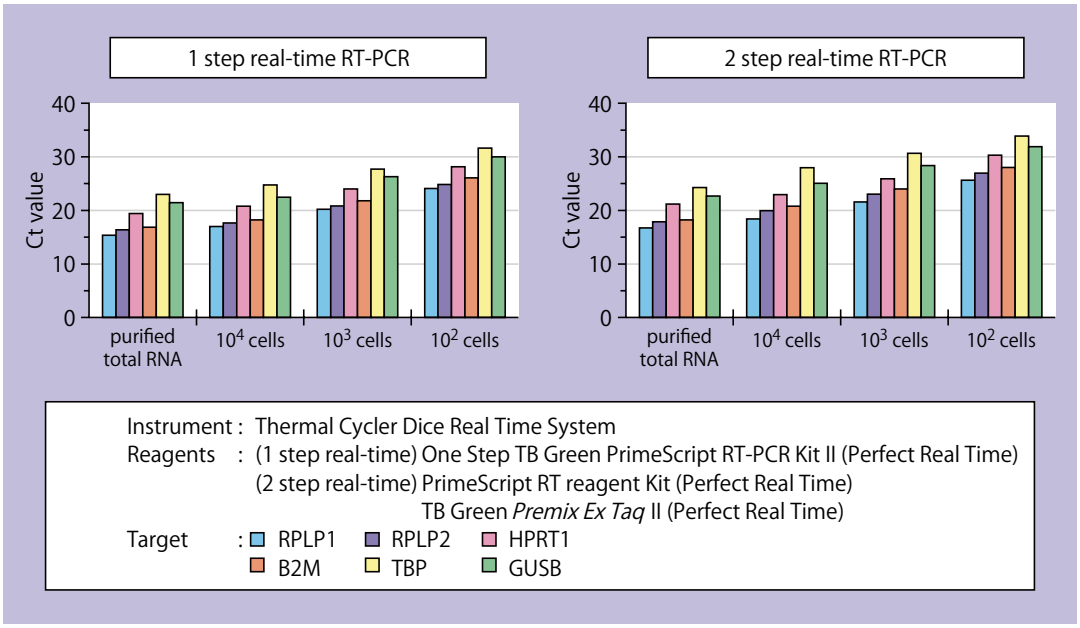


Figure 1. Gene expression analysis.

IX. Troubleshooting

No amplification with real time RT-PCR

- Reconsider PCR primer design. Refer to the One Step TB Green PrimeScript RT-PCR Kit (Perfect Real Time) (Cat. #RR066A/B) or One Step TB Green PrimeScript RT-PCR Kit II (Perfect Real Time) (Cat. #RR086A/B) protocol.
- Depending on cell species or culture conditions, the number of cell or experimental protocol may need to be optimized.
- Wash cells with CellAmp Washing Buffer and remove contaminants in the culture medium. Remove as much of the culture medium and CellAmp Washing Buffer as possible.
- Prepare real time RT-PCR reaction mixture on ice and protect from light until starting the reaction.
- When too much cell lysate volume is used in RT-PCR and reverse transcription, reaction efficiency might be reduced. Reduce the cell lysate volume.

X. Related Products

One Step TB Green® PrimeScript™ RT-PCR Kit (Perfect Real Time) (Cat. #RR066A/B)＊
One Step TB Green® PrimeScript™ RT-PCR Kit II (Perfect Real Time) (Cat. #RR086A/B)
One Step TB Green® PrimeScript™ PLUS RT-PCR Kit(Perfect Real Time) (Cat. #RR096A/B)＊
One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) (Cat. #RR064A/B)
PrimeScript™ RT reagent Kit (Perfect Real Time) (Cat. #RR037A/B)
PrimeScript™ RT Master Mix (Perfect Real Time) (Cat. #RR036A/B)
TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Cat. #RR820A/B)
TB Green® *Premix Ex Taq*™ (Tli RNaseH Plus) (Cat. #RR420A/B)
TB Green® Premix DimerEraser™ (Perfect Real Time) (Cat. #RR091A/B) ＊
Premix Ex Taq™ (Probe qPCR) (Cat. #RR390A/B)
Thermal Cycler Dice™ Real Time System III (Cat. #TP950/TP970/TP980/TP990)＊

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