Cat. # 3731

For Research Use

TaKaRa

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

Product Manual

v201904Da

Table of Contents

I. Description

This kit is a product to prepare template for 1 step real time RT-PCR by simple method without RNA extraction process from cells cultured in 96-well plates or others. This kit was developed to use only for 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) and One Step TB Green PrimeScript PLUS RT-PCR Kit (Perfect Real Time) (Cat. #RR096A/B). By using them together, gene expression analysis can be performed about 2.5 hours (without DNase treatment, 2 hours) .
Templates which prepared directly from small quantities of cell by this kit is useful for analysis of gene expression profile without influencing on high sensitivity of 1 step real time RT-PCR. Because genomic DNA can be removed efficiently by treating DNase in this kit, this kit is powerful for expression analysis of gene, primers of which cannot be designed in both end of the exon junction, or which is expressing at low level.

II. Components*

Cell Washing Buffer	12.5 ml x 2
Cell Processing Buffer	8.0 ml
DNase I for Direct RNA Prep	200 μl
DNase I Buffer for Direct RNA Prep	1.0 ml x 2

＊ This corresponds to 200 wells of 96-well plate.

III. Reagents not Supplied in This Kit

One Step TB Green PrimeScript RT-PCR Kit (Perfect Real Time) (Cat. #RR066A/B)＊1, 2
One Step TB Green PrimeScript RT-PCR Kit II (Perfect Real Time) (Cat. #RR086A/B)＊1
One Step TB Green PrimeScript PLUS RT-PCR Kit (Perfect Real Time) (Cat. #RR096A/B)＊1, 2

＊ 1 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.

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

IV. Storage

-20℃

Cell Washing Buffer and Cell Processing Buffer can be stored at 4℃ after thawing it. Avoid contamination.

V. General Considerations

- Use this kit in combination with 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) or One Step TB Green PrimeScript PLUS RT-PCR Kit (Perfect Real Time) (Cat. #RR096A/B). Compatibility of this kit with other 1 step real time RT-PCR kit or 2 step real time RT-PCR kit is not confirmed.
- If precipitation appear during thawing of Cell Washing Buffer and Cell Processing Buffer, use after be dissolved completely by warming up to room temperature.
- Perform the lysate preparation quickly without taking too much time.

Guidelines for RNA preparation

1. 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 spatel, 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 treat with autoclave (prevent RNA's carboxymethylization cause by DEPC) before using it. It is important to separate equipments for RNA experiment exclusively from other equipments.
2. Reagents should be prepared with 0.1% DEPC treated water as much as possible, and treat with autoclave before use. If reagents which are not autoclave treatable are included, use equipments and water which has been sterilized to prepare the solution, and then perform filter sterilization before use.
3. Extra precautions should be taken during the sample preparation, including use of clean disposable gloves and avoiding unnecessary speaking during assembly to prevent RNase contamination from operator sweat or saliva.

VI. Protocol

VI - 1. Preparation of reagents

Prepare DNase solution on ice.

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

＊1 If other types of plate are used, please refer VII. 2.

VI - 2. Preparation of cell lysate from adherent cultured cells

1) Inoculate appropriate numbers of cells to 96-well plate.＊2
2) Incubate until reaches to appropriate cell numbers or confluent.
3) Remove culture medium as much as possible.
4) Add 125 μl＊3 of Cell Washing Buffer to each well.
5) Remove Cell Washing Buffer as much as possible.
6) Add 40 μl＊3 of Cell Processing Buffer to each well, then incubate for 5 min at room temperature (15 - 28℃) .
7) After pipetting cell lysate in each well several times, and transfer the lysate to PCR tube or microcentrifuge tube. Then incubate for 10 min at 75℃.
8) After cooling down on ice, add 10 μl＊3 of DNase solution to each well and incubate for 20 min at 37℃. (When DNase treatment would not be performed, skip to 9.)
9) Following the protocol described in [VII-1], perform 1 step real time RT-PCR using the prepared lysate as a template. For 25 μl reaction volume of RT-PCR, use less than 2 μl of the lysate. The prepared lysate should be kept on ice and should start real time RT-PCR within 20 min. The lysate can be also stored for about 2 weeks at -80℃.

＊2 See VII-2 about the number of cell inoculated.
＊3 If other types of plate are used, see VII-2.

VI - 3. Preparation of cell lysate from non-adherent cells

1) Count cell numbers and transfer cells less than 1 x 10^4 to microcentrifuge tube.
2) Centrifuge at 300*g* for 5 min.
3) Remove culture medium as much as possible.
4) Add 125 μl＊4 of Cell Washing Buffer.
5) Centrifuge at 300*g* for 5 min.
6) Remove Cell Washing Buffer as much as possible.
7) Add 40 μl＊4 of Cell Processing Buffer and incubate for 5 min at room temperature (15 - 28℃) .
8) Incubate for 10 min at 75℃.
9) After cooling down on ice, add 10 μl＊4 of DNase solution to each tube and incubate for 20 min at 37℃. (When DNase treatment would not be performed, skip to step 10.)
10) Following the protocol described in [VII-1], perform 1 step real time RT-PCR using the prepared lysate as a template. For 25 μl reaction volume of RT-PCR, use less than 2 μl of the lysate. The prepared lysate should be kept on ice and should start real time RT-PCR within 20 min. The lysate can be also stored for about 2 weeks at -80℃.

＊4 When using cell that exceeds 1 x 10^4 cells, use more reagents proportionally.

VII. Appendix

VII-1. Experimental example with Thermal Cycler Dice Real Time System //

1. Add 1 - 2 μl of cell lysate to a reaction PCR tube or 96-well plate on ice.
2. Prepare master mixture shown below on ice.

2 - a) In case of One Step TB Green PrimeScript RT-PCR Kit II (Perfect Real Time) (Cat. #RR086A/B)

< For 1 reaction >

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	1.0 μl	0.4 μM*2
PCR Reverse Primer (10 μM)*1	1.0 μl	0.4 μM*2
RNase Free dH_2O	7.5 - 8.5 μl	
Total	23 - 24 μl	

＊1 Do not mix primers and cell lysate directly. Primers might be digested by DNase activity remained in the cell lysate.
＊2 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.

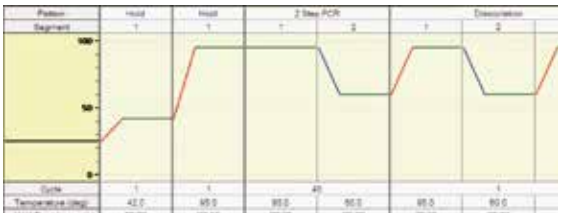
2 - b) One Step TB Green PrimeScript RT-PCR Kit (Perfect Real Time) (Cat. #RR066A/B)

< For 1 reaction >

Reagent	Volume	Final Conc.
2X One Step TB Green RT-PCR Buffer III	2.5 μl	1X
TaKaRa Ex Taq® HS (5 U/μl)	0.5 μl	
PrimeScript RT enzyme Mix II	0.5 μl	
PCR Forward Primer (10 μM)*3	0.5 μl	0.2 μM*4
PCR Reverse Primer (10 μM)*3	0.5 μl	0.2 μM*4
RNase Free dH_2O	8.5 - 9.5 μl	
Total	23 - 24 μl	

＊3 Do not mix primers and cell lysate directly. Primers might be digested by DNase activity remained in the cell lysate.
＊4 The final concentration of primers can be 0.2 μM in most reactions. When it does not work, determine the optimal concentrations within the range of 0.1 - 1.0 μM.

3. Add master mix to the cell lysate in PCR tube or 96-well plate and mix well. After centrifuging PCR tube or plate briefly, set on Thermal Cycler Dice Real Time System *II* and start reaction.
   It is recommended to perform the reaction by following the protocol in below. First try this protocol, and then adopt the PCR reaction condition as needed. For primer which has lower Tm value might be difficult perform the reaction with shuttle PCR, in such case, perform 3 step PCR.



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

Note：This product combines the high performance of *TaKaRa Ex Taq* HS, which is an enzyme for hot start PCR utilizing Taq antibody. Initial denaturation step prior to PCR should be at 95℃ for 10 sec. No need to heat at 95℃ for (5 -) 15 min as the initial denaturation, required for chemically modified Taq polymerase. If longer heat treatment is done, the enzyme activity decreases and the amplification efficiency and the accuracy in quantification can also be affected.

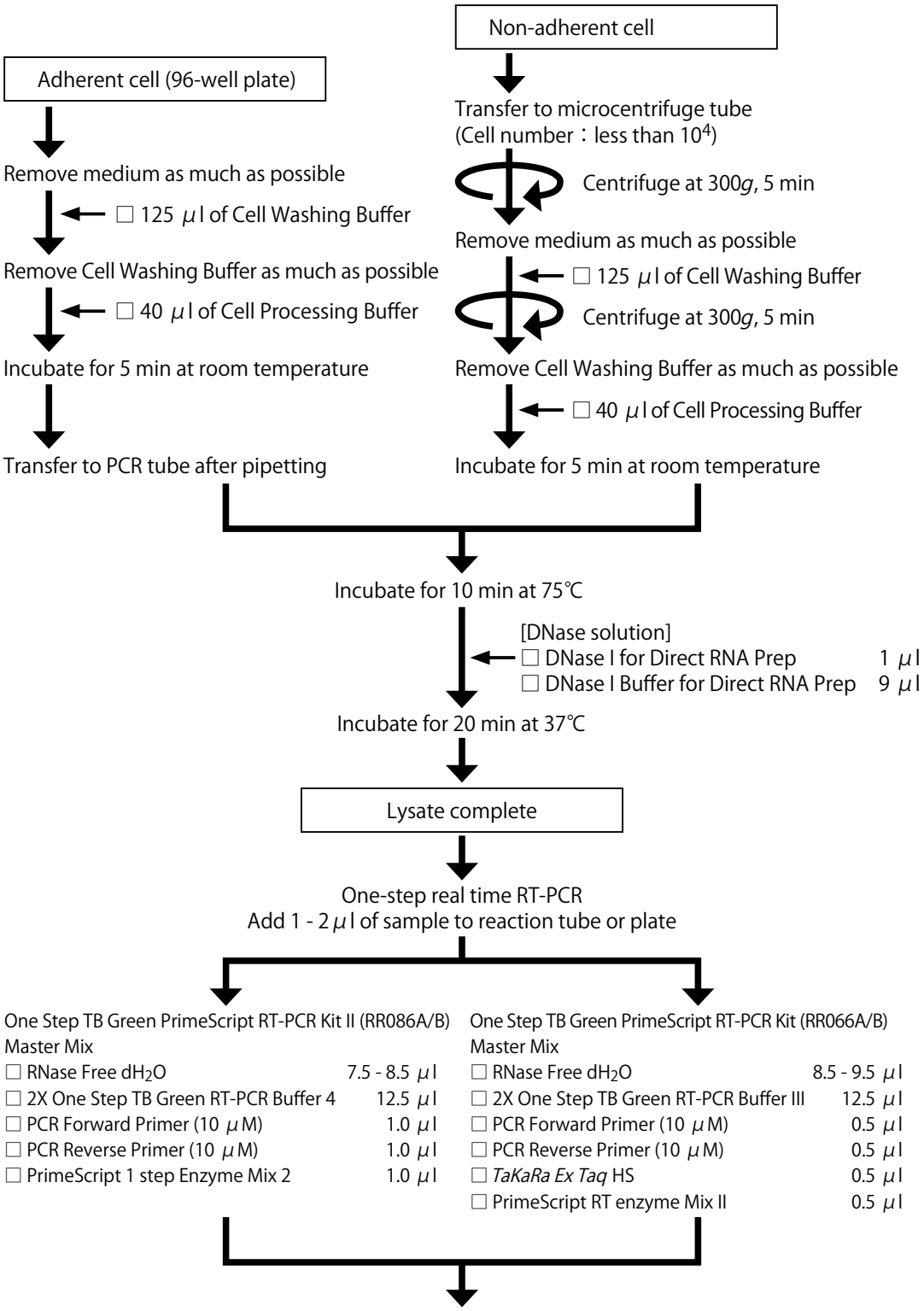
4. After completing reaction, verify amplification curve and dissociation curve.

VII-2. Cell number of adherent cell and quantities of each reagents per well of culture plate used.

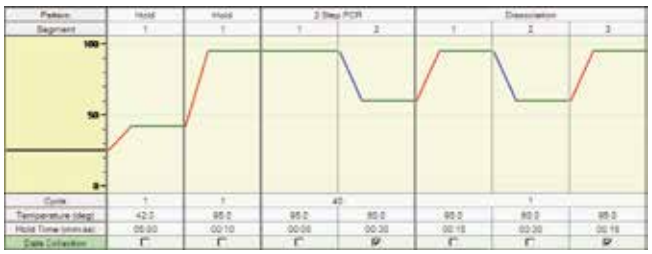
	96-well	48-well	24-well	12-well	6-well
Cell number inoculated (cells/well)＊	1 x 10^4	2 x 10^4	4 x 10^4	8 x 10^4	2 x 10^5
Cell Washing Buffer	125 µl	250 µl	500 µl	1 ml	2.5 ml
Cell Processing Buffer	40 µl	80 µl	160 µl	320 µl	800 µl
DNase I for Direct RNA Prep	1 µl	2 µl	4 µl	8 µl	20 µl
DNase I Buffer for Direct RNA Prep	9 µl	18 µl	36 µl	72 µl	180 µl

＊ This value is when using general adherent cell and cultured condition. Depending on cells or culture condition used, number of cells inoculated and experimental protocol should be evaluated.

CellAmp Direct RNA Prep Kit Flow Chart



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 are completed

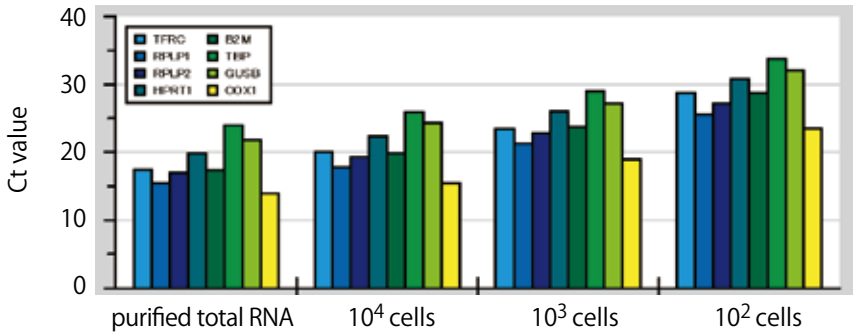
VIII. Experimental Example：Analysis of gene expression profiling

［Method］

HeLa cell was inoculated to 1 x 10^4, 1 x 10^3, or 1 x 10^2 cells/well in 24 well plate. After cultured for 72 hours, cell lysate were prepared by following the protocol to be template. Using 8 kinds of genes as targets, gene expression analysis were performed with 1 step real time RT-PCR. For experimental control, purified total RNA (100 ng) was used.

［Result］

When gene expression analysis was performed with the 8 kinds of genes, stable gene expression profile that was similar to when using purely prepared RNA was obtained from each of different cell numbers.



Instrument： Thermal Cylcer Dice Real Time System
Reagents： One Step TB Green PrimeScript RT-PCR Kit II (Perfect Real Time)
Target： Human TFRC, RPLP1, RPLP2, HPRT1, B2M, TBP, GUSB, COX1

Figure. Result of gene expression analysis targeting 8 kinds of gene

IX. Troubleshooting

No amplification with real time RT-PCR

- Reconsider PCR primer design. To perform real time RT-PCR efficiently, it is important to design PCR primer with good reactivity. For PCR primer design, refer [Guideline for designing of primer] at the protocol of 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) or One Step TB Green PrimeScript PLUS RT-PCR Kit (Perfect Real Time) (Cat. #RR096A/B).
- Depending on cell species or culture condition, numbers of cell or experimental protocol should be evaluated.
- Wash cell with Cell Washing Buffer and remove contaminants in the culture medium. Furthermore, remove the culture medium and Cell Washing Buffer as much as possible.
- Prepare 1 step real time RT-PCR reaction mixture on ice. Store on ice in the condition protected from light until starting the reaction.
- When volumes of cell lysate added to RT-PCR is too much, reaction efficiency might be reduced. Reduce the cell lysate added.
- Do not mix primers and cell lysate directly. Primers might be digested by DNase activity remained in the cell lysate.

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)＊
Thermal Cycler Dice™ Real Time System *II* (Cat. #TP900)＊

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