

For Research Use

---

**TAKARA**

**Retrovirus Titer Set  
(for Real Time PCR)**

---

Product Manual

## Table of Contents

I.	Description.....	3
II.	Components.....	3
III.	Storage.....	4
IV.	Precautions .....	4
V.	Procedure .....	5
VI.	Applications.....	9
VII.	Appendix.....	10
VIII.	Related Products.....	11

## I. Description

The Retrovirus Titer Set is designed to quantitate viral RNA transcribed from retrovirus vectors based on the murine leukemia virus (MLV). The kit is based on real-time RT-PCR method to quantitate MLV RNA and accurately determine the virus titer. Use of this kit with the One Step TB Green® PrimeScript™ RT-PCR Kit (Cat. #RR066A), One Step TB Green PrimeScript RT-PCR Kit II (Perfect Real Time) (Cat. #RR086A) provides a direct measurement of RNA titer from virus supernatant in only about 4 hours. In addition, RNA titers of almost all MLV-based retrovirus vector can be measured because using the MLV packaging signal region as an amplification target.

The conventional method for measuring the infection titer of a retrovirus vector involves calculating the biological titer by infecting an indicator cell with the retrovirus and using fluorescence or drug resistance to measure infection efficiency. This method requires several days to complete, and cannot be used to measure a retroviral vector that lacks a marker or drug-resistance gene. In addition, when determined multiplicity of infection (MOI) based on biological titer, the procedure requires several days; thus, the virus supernatant must be kept frozen until infection. Freeze/thaw cycles can lower the virus titer and alter the MOI, and additional manipulations increase the likelihood of contamination.

This kit can measure retrovirus titer without marker or drug-resistance genes because it quantitates retroviral vector RNA. Because results can be obtained within about 4 hours, infection at the obtained MOI is possible without freezing the virus supernatant.

The One Step TB Green PrimeScript RT-PCR Kit (Perfect Real Time), which is an intercalator-based one-step real-time RT-PCR system is recommended for use with the Retrovirus Titer Set.

This kit combines PrimeScript reverse transcriptase, which has excellent fidelity and a rapid elongation rate, with *Takara Ex Taq*® HS polymerase, a highly efficient, specific PCR enzyme.

## II. Components (for 100 reactions; 25 µl reaction system) \*1

Forward Titer Primer FRT-1 (10 pmol/µl)	50 µl
Reverse Titer Primer RRT-1 (10 pmol/µl)	50 µl
RNA Control Template (5 x 10 <sup>8</sup> copies/µl)	10 µl
EASY Dilution (for Real Time PCR)	1 ml x 2
DNase I (RNase Free) (5 U/µl)	40 µl
10X DNase I Buffer	50 µl
RNase Inhibitor (40 U/µl)	10 µl

\*1 For Real Time RT-PCR (100 reactions) and DNase I treatment (20 reactions).

### Reagents required (not supplied in the kit)

- One Step TB Green PrimeScript RT-PCR Kit (Perfect Real Time) (Cat. #RR066A)\*2, 3 or One Step TB Green PrimeScript RT-PCR Kit II (Perfect Real Time) (Cat. #RR086A)\*2
- RNase-Free Water

\*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.

\*3 Not available in all geographic locations. Check for availability in your area.

**III. Storage**

-20°C

**Note:** RNA Control Template should be divided into small aliquots upon receipt to avoid multiple freeze/thaw cycles, and stored at -70 to -80°C.

**IV. Precautions**

Please read these instructions before using the kit.

- (1) Preparation of a Master Mix (containing RNase Free dH<sub>2</sub>O, buffer, and enzymes) is recommended to allow accurate dispensing of reagents, minimize pipetting losses, and avoid repeated dispensing of each reagent. This step helps to minimize variability between experiments.
- (2) *Takara Ex Taq* HS and PrimeScript RT Enzyme Mix II (components of the One Step TB Green PrimeScript RT-PCR Kit), or PrimeScript 1step Enzyme Mix 2 (a component of the One Step TB Green PrimeScript RT-PCR Kit II) should be gently spun down to collect the solution in the bottom of tube before use. Pipet enzymes carefully due to the viscosity of the 50% glycerol. Store the enzyme at -20°C until just before use and return it to the freezer promptly.
- (3) A precipitate may be observed in the 2X One Step TB Green RT-PCR Buffer III (a component of the One Step TB Green PrimeScript RT-PCR Kit), or 2X One Step TB Green RT-PCR Buffer 4 (a component of the One Step TB Green PrimeScript RT-PCR Kit II) upon thawing. This precipitate can be completely dissolved by vortexing, and after resuspension will not affect the performance of the reaction.
- (4) Use new disposable pipette tips for transferring reagents to avoid contamination among samples.

**V. Procedure**

Read carefully the product manual for the One Step TB Green PrimeScript RT-PCR Kit or the One Step TB Green PrimeScript RT-PCR Kit II and instructions for your thermal cycler before beginning this procedure.

**1. Preparing virus supernatant for titer measurement**

When cells are initially transfected with a retrovirus, a large amount of plasmid which is used with transfection is present in the virus supernatant. Therefore, it become a cause to bring high background if this supernatant is used directly for real-time RT-PCR. After stable integration of the viral genome, DNase I treatment is generally not required; however, background may still be high due to elution of genomic DNA during virus vector preparation. For these reasons, DNase I treatment is recommended to include in the procedure in the case.

1-1. Prepare the reaction mixture shown below\* 1

<Per reaction>

Reagent	Volume
Virus supernatant	12.5 $\mu$ l
10X DNase I Buffer	2.5 $\mu$ l
DNase I (RNase Free) (5 U/ $\mu$ l)	2.0 $\mu$ l
RNase Inhibitor (40 U/ $\mu$ l)	0.5 $\mu$ l
RNase-Free Water	7.5 $\mu$ l
Total	25 $\mu$ l

- \* 1 Reagents for 20 DNase I treatments are included in this kit. Additional reagents may be obtained from Takara Bio USA as follows:  
Recombinant DNase I (RNase-free) (Cat. #2270A)\*2
- \* 2 10X DNase I Buffer included  
Recombinant RNase Inhibitor (Cat. #2313A)

1-2. Incubate the reaction mixture as follows:

37°C 30 min  
70°C 10 min

**2. Preparing the sample for an standard curve**

There are two methods to create an standard curve.

- **Creating a Standard Curve using the RNA Control Template**  
 Perform real-time RT-PCR using the RNA Control Template provided in this kit and plot an standard curve. The virus RNA titer can be calculated from this standard curve. In this case, the calculated value corresponds to the retrovirus RNA that is present in the supernatant.  
 To obtain the biological titer of a virus sample, measure the RNA titer of a retrovirus vector sample whose biological titer is known (the viral vector backbone should be the same as the test sample, and be prepared by the same method) at the same time. Compare this value with the RNA titer obtained from the standard curve to determine the test sample's biological titer.
- **Using retrovirus vector whose biological titer has already been measured**  
 Perform real-time RT-PCR by using serially diluted virus samples whose biological titer has already been measured, then calculate the test sample's virus titer from the standard curve. The virus samples used for creating the standard curve and the test sample virus must have the same vector backbone and be prepared by the same methods.

2-1. Preparing the standard curve by using RNA Control Template\*<sup>1</sup>

1. Mix the reagents in the quantities shown for Tube 1, resulting in a 1 : 5 dilution of the RNA Control Template.
2. Pipet 18  $\mu$ l of EASY Dilution into Tubes 2 - 6.
3. Transfer 2  $\mu$ l from Tube 1 to Tube 2, and mix well.
4. Repeat for the remaining tubes, each time adding 2  $\mu$ l of the RNA solution from the previous tube.
5. Proceed to Step 3 below.

Tube	RNA solution ( $\mu$ l)	EASY Dilution ( $\mu$ l)	Copies/ $\mu$ l
1	2	8	$1 \times 10^8$
2	2	18	$1 \times 10^7$
3	2	18	$1 \times 10^6$
4	2	18	$1 \times 10^5$
5	2	18	$1 \times 10^4$
6	2	18	$1 \times 10^3$

2-2. Preparing the standard curve by using retrovirus vector whose biological titer has already been measured

Prepare serial dilutions of retrovirus vector whose biological titer has already been measured (see Part VI, experiment 2 for an example).\*<sup>2</sup>

- \* 1 You may need to prepare additional serial dilutions to obtain an standard curve in the same range as the test sample.
- \* 2 It is recommended to use the same medium that was used during preparation of retrovirus vector to prepare serial dilutions.

**3. Preparing real-time RT-PCR reagents (using One Step TB Green PrimeScript RT-PCR Kit)**

Prepare the following reagents on ice.\*1

< Per reaction > \*2

Reagent	Volume	Final conc.
2X One Step TB Green RT-PCR Buffer III *3	12.5 $\mu$ l	1X
Takara Ex Taq HS (5 U/ $\mu$ l) *3	0.5 $\mu$ l	
PrimeScript RT Enzyme Mix II *3	0.5 $\mu$ l	
Forward Titer Primer FRT-1 (10 pmol/ $\mu$ l)	0.5 $\mu$ l	0.2 $\mu$ M
Reverse Titer Primer RRT-1 (10 pmol/ $\mu$ l)	0.5 $\mu$ l	0.2 $\mu$ M
Virus supernatant or sample for preparing standard curve *4,5	2 $\mu$ l	
RNase Free dH <sub>2</sub> O *3	8.5 $\mu$ l	
<b>Total</b>	<b>25 <math>\mu</math>l</b>	

- \* 1 The reaction conditions shown are optimized for the Thermal Cycler Dice™ Real Time System // (Cat. #TP900). If using other thermal cyclers, refer to the instruction manual for optimal conditions.
- \* 2 It is recommended to perform two or more reactions per sample for virus supernatant or samples for creating standard curve to obtain reproducible data.
- \* 3 Component of the One Step TB Green PrimeScript RT-PCR Kit (Cat. #RR066A).
- \* 4 Perform DNase I treatment for retrovirus vectors samples as described in Step 1.
- \* 5 Use diluted virus supernatant to avoid interference from components in the culture medium. It is also recommended to determine a suitable dilution range by testing several orders of magnitude (see Part VI, experiment 1 for an example).

**4. Thermal cycling\***

After centrifuging the reaction tubes or plate, program the Thermal Cycler Dice Real Time System // as follows:

- Stage 1 : Reverse transcription
  - Hold
  - 42°C 5 min
  - 95°C 10 sec
- Stage 2 : PCR
  - Cycle: 40
  - 95°C 5 sec
  - 60°C 30 sec (Data Collection ; FAM)
- Stage 3 : Dissociation

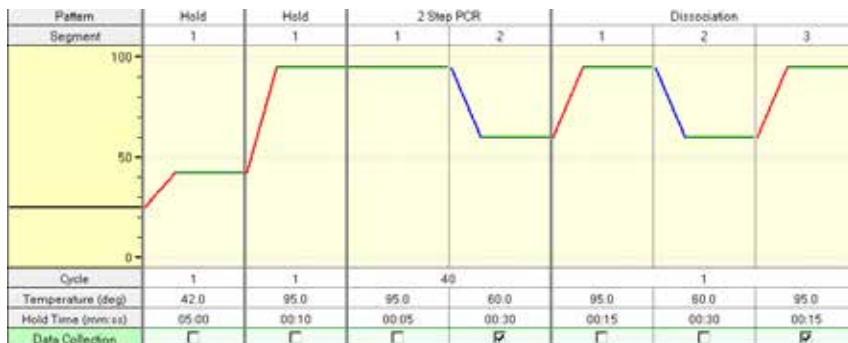


Figure 1. Thermal Profile Setup Monitor

\* The reaction conditions shown are optimized for the Thermal Cycler Dice Real Time System // (Cat. #TP900). If using other thermal cyclers, refer to the instruction manual for optimal conditions.

**5. Analyzing the results**

After the reaction is complete, confirm the amplification conditions, then plot the standard curve and quantify the test sample.



VI. Applications

Experiment 1. Inhibition effect of retrovirus culture medium

[ Procedure ]

Retrovirus-producing cells were cultured in two batches: using serum-free medium (GT-T-Retro I) and serum-containing medium (DMEM, 10% FBS). For each batch, the retroviral vector samples were analyzed at the original concentration, and at dilutions of 1 : 2, 1 : 4, 1 : 8, 1 : 16, and 1 : 32. Real-time RT-PCR was performed as described above, using the same culture medium to prepare serial dilutions for analysis.

[ Results ]

The relationship between dilution and Ct value is shown in Figure 2 (serum-free medium) and Figure 3 (serum-containing medium). No significant interference of serum was observed.

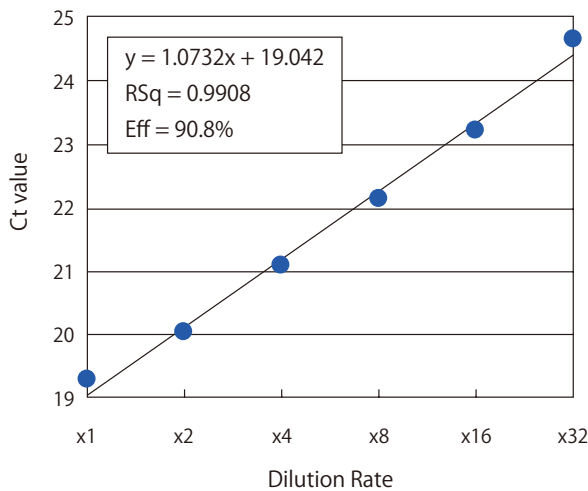


Figure 2. Retrovirus vector prepared by using serum-free medium (GT-T-Retro I)

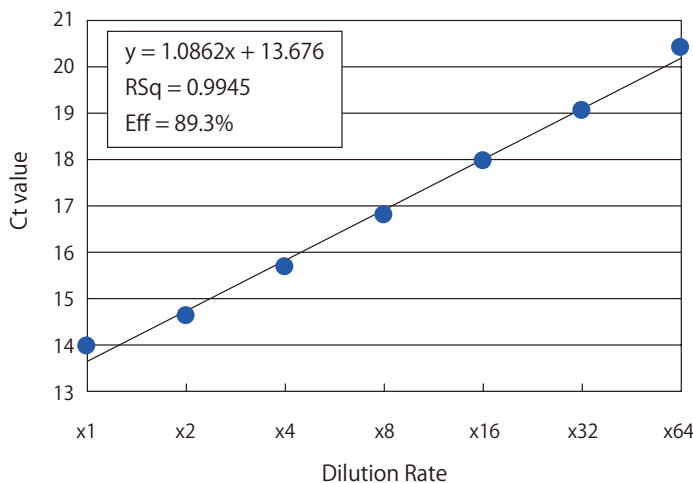


Figure 3. Retrovirus vector prepared by using serum medium (DMEM, 10% FBS)

**Experiment 2. Plotting an standard curve using retrovirus vector of known biological titer**

[ Procedure ]

HT1080 cells were infected with a ZsGreen expression retroviral vector in serum-containing medium (DMEM, 10% FBS). The biological titer ( $1.82 \times 10^6$  ivp/ml) was calculated by fluorescence-activated cell sorting (FACS) analysis. Next, serial dilutions (1 : 10, 1 : 100, and 1 : 1,000) were prepared using the same culture medium. After treating with DNase I, real-time RT-PCR was performed as described above, and the standard curve was plotted.

[ Results ]

As shown in Figure 4, a highly precise analytical curve was obtained.

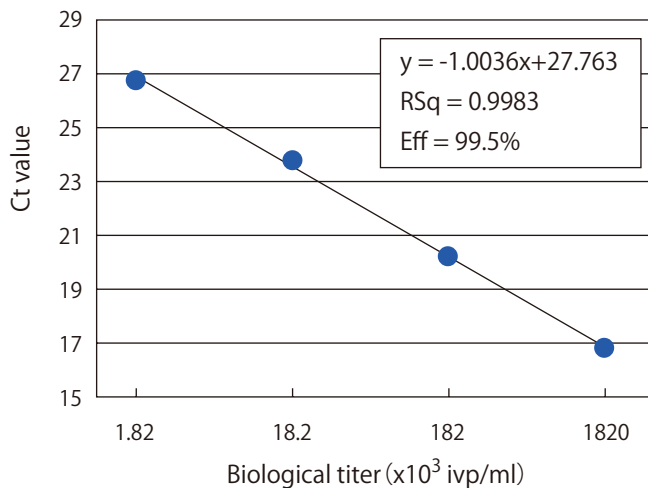


Figure 4. Analytical curve with retrovirus vector which biological titer has been.

**VII. Appendix**

**Handling the RNA Control Template**

To obtain a precise standard curve, the reactions containing the RNA Control Template must be carefully performed. Thus, it is essential to prevent contamination with RNase derived from equipment and solutions used. Extra precautions should be taken during sample preparation, such as using clean, disposable gloves, dedicating a work area to exclusive use for RNA preparation, and avoiding unnecessary talking during the procedure to prevent RNase contamination from saliva.

**VIII. Related Products**

- [ Real Time PCR instrument ]
  - Thermal Cycler Dice™ Real Time System // (Cat. #TP900)\*
  - Thermal Cycler Dice™ Real Time System *Lite* (Cat. #TP700)\*
  
- [ Eight connected tube and cap ]
  - 0.2 ml Hi-8-Tube (Cat. #NJ300)
  - 0.2 ml Hi-8-Flat Cap (Cat. #NJ302)
  
- [ High-efficiency gene transfer enhancer for retrovirus vectors ]
  - RetroNectin® (Recombinant Human Fibronectin Fragment) (Cat. #T100A/B)
  
- [ Retrovirus packaging kit ]
  - Retrovirus Packaging Kit Eco/Ampho (Cat. #6160/6161)
  
- [ Retroviral vector DNA ]
  - pDON-AI-2 Neo DNA/pDON-AI-2 DNA (Cat. #3653/3654)
  - pMEI-5 Neo DNA/pMEI-5 DNA (Cat. #3655/3656)

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

TB Green, *Takara Ex Taq*, and RetroNectin are registered trademarks of Takara Bio Inc. PrimeScript and Thermal Cycler Dice are trademarks of Takara Bio Inc.

---

**NOTE:** This product is for research use only. It is not intended for use in therapeutic or diagnostic procedures for humans or animals. Also, do not use this product as food, cosmetic, or household item, etc.

Takara products may not be resold or transferred, modified for resale or transfer, or used to manufacture commercial products without written approval from Takara Bio Inc.

If you require licenses for other use, please contact us by phone at +81 77 565 6972 or from our website at [www.takara-bio.com](http://www.takara-bio.com).

Your use of this product is also subject to compliance with any applicable licensing requirements described on the product web page. It is your responsibility to review, understand and adhere to any restrictions imposed by such statements.

All trademarks are the property of their respective owners. Certain trademarks may not be registered in all jurisdictions.

---