Takara Bio USA

Retro-X™ qRT-PCR Titration Kit User Manual

Cat. No. 631453
(050919)

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I. Introduction

A. Summary

The Retro-X qRT-PCR Titration Kit provides a fast and simple method for titrating retroviral stocks. The kit employs a quick RNA purification step and determines viral RNA genome content using qRT-PCR and TB Green® technologies. Titration can be completed in only 4 hours. The Retro-X qRT-PCR Titration Kit is compatible with MMLV-based retroviruses or retroviral vectors carrying unmodified MMLV-derived packaging signal, which is present in the backbone of the majority of Takara Bio's retroviral vectors (like pQCXIN or pLXSN). A significantly modified packaging signal, like the one carried by MSCV vectors, would be incompatible with the kit. Annotated maps and nucleotide sequences of Takara Bio's retroviral vectors can be accessed from the TBUSA website at www.takarabio.com. Using this kit reduces time delays between virus harvest and target cell infection, allowing you to do both on the same day. Delays and freeze-thaw cycles that reduce virus infectivity can be avoided. Target cells can be infected at a known multiplicity of infection (MOI) for more consistent results.

B. Protocol Overview

Viral supernatant is collected from your virus-producing cell line and centrifuged to remove cells and debris. Using the NucleoSpin RNA Virus kit, genomic viral RNA is purified from a small aliquot of the supernatant. The RNA is then treated with DNase I to remove any residual plasmid DNA that may have been carried over from transient transfection of the packaging cells. Serial dilutions of the viral RNA sample are subjected to qRT-PCR to determine threshold cycle (Ct) values for each dilution. Titration by qRT-PCR is also compatible with the ROX qPCR reference dyes provided with the kit (optional). The RNA genome copy number in a sample dilution is determined by finding the copy number that corresponds to its Ct value on a standard curve generated from serial dilutions of the calibrated Retro-X RNA Control Template.

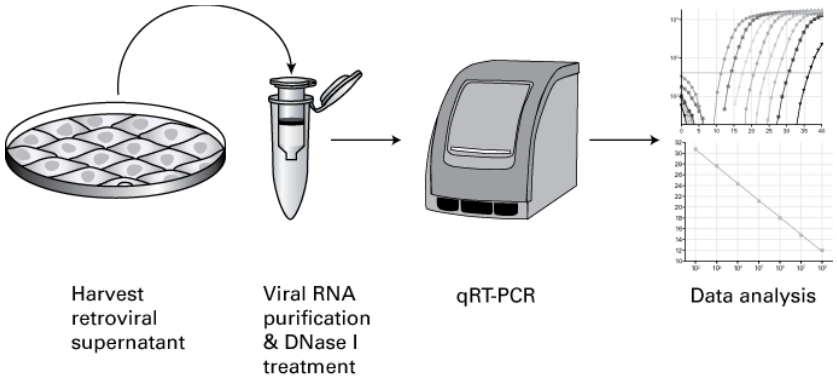

Figure 1. Flowchart of the procedures for titrating retroviral supernatants with the Retro-X qRT-PCR Titration Kit.

C. Correlating RNA Titer with Infectivity

The first time you have performed the qRT-PCR titration to determine RNA copy number, you may also wish to determine an IFU value by independent means (e.g., via FACS or colony selection) in order to establish a relationship between the two values. Representative ratios between different titration values for typical Retro-X expression systems are shown in Table I. For subsequent, similarly prepared virus supernatants, the qRT-PCR titration value may be used as a reference to determine a relative IFU value for the supernatant and the MOI for the infection experiment (Carmo et al. 2004). Users should establish correlation values for each different retrovirus or packaging method used.

Table I. Correlating Virus Titration Methods[1]

				Titration Ratios	
Packaging Method	**Flow Cytometry (IFU/ml)**	**G418 (cfu/ml)**	**qRT-PCR (copies/ml)**	**RT:FACS (copies/IFU)**	**RT:G418 (copies/cfu)**
Transient Transfection[2]	1.1×10^7	2.9×10^6	6.3×10^9	584	2,176
Stable Producer Cell Line	2.1×10^8	6.8×10^7	2.4×10^{11}	1,135	3,505

[1]To determine infectivity titers, NIH 3T3 cells were infected with qRT-PCR-titrated supernatants containing an amphotropic retrovirus harboring a ZsGreen1 expression construct and a neomycin resistance gene. For G418 resistance, cells were selected in G418 for 12 days and resistant colonies were stained and counted. For titration by flow cytometry, cells were harvested 48 hr post infection and analyzed using a BD FACSCalibur flow cytometer.

[2]Retro-X Universal Packaging System

II. List of Components

Store the Retro-X RNA Control Template at –70°C.

Store all other components at –20°C.

Box 1: Retro-X qRT-PCR Titration Components (Cat. No. 631454)
(not sold separately)

- 30 µl Retro-X RNA Control Template (5 x 10^8 copies/µl)
- 80 µl DNase I (5 units/µl)
- 50 µl DNase I Buffer (10X)
- 100 µl Retro-X Forward Titer Primer (10 µM)
- 100 µl Retro-X Reverse Titer Primer (10 µM)
- 4 tubes EASY Dilution Buffer (1 ml/tube)

Box 2: NucleoSpin RNA Virus kit (10 preps; Cat. No. 740956.10)
See the NucleoSpin RNA Virus & Virus F User Manual for a list of components and storage conditions.

Box 3: Quant-X™ One-Step qRT-PCR TB Green Kit (200 rxns; Cat. No. 638317; not sold separately)

- 3 tubes 2X Quant-X Buffer (0.84 ml per tube)
- 100 µl Quant-X Enzyme (5 U/µl)
- 100 µl RT Enzyme Mix
- 2 tubes RNase-Free Water (1.25 ml per tube)
- 100 µl 50X ROX Reference Dye LSR
- 100 µl 50X ROX Reference Dye LMP

Product Documents

Documents for our products are available for download at takarabio.com/manuals
The following documents apply to this product:

- Retro-X qRT-PCR Titration Kit User Manual (PT3952-1)
- Retro-X qRT-PCR Titration Kit Protocol-at-a-Glance (PT3952-2)
- NucleoSpin RNA Virus & Virus F User Manual
- Quant-X™ One-Step qRT-PCR TB Green Kit User Manual (PT5058-1)
- Quant-X™ One-Step qRT-PCR TB Green Kit Protocol-at-a-Glance (PT5058-2)

III. Additional Materials Required

- Work areas and pipettors free of contaminating DNA, RNA, and nucleases.
- Quantitative real-time PCR Thermocycler (e.g. Mx3000P, Stratagene; ABI 7300, ABI 7500, or ABI 7900, Applied Biosystems; or equivalent)
- 96-well PCR plates and 8-well PCR strips
- Repeating pipettor with 23 µl capacity (Section IV.C)
- Multichannel pipettor(s) with 2–25 µl capacity

IV. Retroviral Titration Protocols

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING.
Successful titration results depend on performing the following steps in sequence.

A. Good PCR Practices

Due to the tremendous amplification power and sensitivity of qPCR, even trace amounts of contaminating DNA and RNA will be amplified and will affect Ct and final copy number values. Before you begin, prepare work areas free of potentially contaminating DNA, RNA, and nucleases. Ideally, dilute samples and controls in one work area with a dedicated set of pipettors and assemble the RT-PCR reactions in a separate area or noncirculating containment hood using a different set of dedicated pipettors. Wear gloves at all times and use PCR pipette tips with hydrophobic filters, and dedicated solutions. We also recommend setting up negative template control (NTC) reactions lacking any template. Finally, perform all post-PCR analyses in a separate area, preferably in a separate room, with different pipettors.

B. Protocol: Purifying Retroviral Genomic RNA

1. Harvest retroviral supernatant from cells and centrifuge 5 min at 2,000 rpm to remove cells and debris.
2. Aliquot 200 µl of supernatant for immediate titration, or store at –80°C.
3. Purify RNA from 150 µl of supernatant using the NucleoSpin RNA Virus Kit according to the protocols contained in the NucleoSpin RNA Virus & Virus F User Manual. Smaller volumes (5–100 µl) can also be used if necessary.

   ATTENTION: Omit the initial 70°C incubation of the sample in the NucleoSpin RNA Virus & Virus F User Manual. Our protocol uses cell-free media supernatant as a sample for viral RNA isolation. Since the sample is cell-free, virus is completely lysed by buffer RAV1 and does not require incubation at 70°C. Hence, this step can be omitted from our protocol. However, if you have performed 70°C incubation during the lysis step of the viral RNA isolation protocol, it should not cause any problems.

4. Elute the RNA in 50–100 µl RNase-free water.
5. If your retrovirus was produced from a stable, retrovirus-producing cell line and not from a transient transfection, DNase I treatment (shown below) is optional, and you may proceed directly to qRT-PCR (Section C). If your retrovirus was produced from a transiently transfected packaging cell line, residual plasmid DNA must be removed prior to qRT-PCR. Treat these types of viral RNA sample(s) with a DNase I reaction as shown in Table II:

Table II. DNase I Reaction

Reagent[1]	Volume (µl)
RNA Sample	12.5
DNase I Buffer (10X)	2.5
DNase I (5 units/µl)	4.0
RNase-Free Water	6.0
Total	**25.0**

6. Combine reagents, mix, and incubate at 37°C x 30 min, followed by 70°C x 5 min. A thermocycler should be used for this reaction. Store the tubes on ice until ready to perform qRT-PCR (Section C).

C. Protocol: qRT-PCR Amplification of Retroviral Genomic RNA

1. **In your reaction assembly work area,** and on ice, assemble a sufficient volume of Master Reaction Mix (MRM) using the reagents shown in Table III. Add the RT Enzyme Mix last. *To ensure that you will have enough MRM, prepare approximately 10% more than the minimum required for your experiment.* Each control, no-template control (NTC), and sample reaction should be performed in duplicate:

Table III. Master Reaction Mix for qRT-PCR

Reagent	Volume/well (µl)	Total wells	Total volume	Total + 10%
RNase-Free Water	8.5 (8.0)			
Quant-X Buffer (2X)	12.5			
Retro-X Forward Primer (10 µM)	0.5			
Retro-X Reverse Primer (10 µM)	0.5			
ROX Reference Dye LSR or LMP (50X)[1]	(0.5)			
Quant-X Enzyme	0.5			
RT Enzyme Mix	0.5			
Total	**23.0**			

[1] The kit is supplied with two different ROX formulations that allow you to normalize fluorescence signals on instruments that are equipped with this option. ROX Reference Dye LSR is for instruments whose excitation source is a 488 nm laser, while ROX Reference Dye LMP is for instruments whose excitation source is either a lamp or an LED. *Be certain to use the formulation that is appropriate for your real-time instrument!*

Example of a basic experiment with minimum MRM volumes:

- Controls: 5 serial dilutions in duplicate; 10 wells x 23 µl = 230 µl MRM.
- NTCs: 3 each in duplicate; 6 wells x 23 µl = 138 µl MRM.
- Samples: 4 dilutions in duplicate; 8 wells x 23 µl = 184 µl MRM/sample.

2. **In your sample dilution work area,** and using PCR grade 8-well strips, construct a standard curve of Retro-X RNA Control Template dilutions, and make serial dilutions of your purified viral RNA sample(s) as described below and shown in Table IV.

3. Add EASY Dilution Buffer to the appropriate wells of the strips, as shown in Table IV. NTCs in Wells 6–8 of Strip 1 contain only EASY Dilution Buffer.

4. In Wells 1–5 of Strip 1, prepare 10-fold serial dilutions of the Retro-X RNA Control Template as follows:
    a. In Well 1, dilute 2 µl of the Retro-X RNA Control Template stock into 18 µl of buffer for a 1:10 dilution (10X stock = 5 x 10^8 copies/µl).
    b. In Wells 2–5, perform 10-fold serial dilutions of the diluted control template in Well 1 by transferring 3 µl of Well 1 into the 27 µl of buffer in Well 2. Repeat similar dilutions for Wells 3–5.
5. Serially dilute your viral RNA samples as shown in Table IV. Each 8-well strip can be used for 2 samples at 4 different concentrations each.
    a. The first well in each series (Wells 1 & 5) should contain 20 µl of undiluted sample (1X).
    b. Subsequent 10-fold sample dilutions (Wells 2–4 & Wells 6–8) can be made by serially transferring 3 µl of the preceding dilution into 27 µl of buffer in the next well.
6. Mix well by tapping gently and centrifuge the strips at 2,000 rpm (4°C) for 1 min to remove any bubbles.

Table IV. Control and Sample Dilutions for qRT-PCR[1]

	Strip 1: Controls			Strip 2+: Samples		
Well	**EASY**	**Additive[2]**	**Amount[3]**	**EASY**	**Additive[2]**	**Amount**
1	18	2	5 x 10^7	–	20	Sample 1: 1X
2	27	3	5 x 10^6	27	3	0.1X
3	27	3	5 x 10^5	27	3	0.01X
4	27	3	5 x 10^4	27	3	0.001X
5	27	3	5 x 10^3	–	20	Sample 2: 1X
6	10	–	NTC	27	3	0.1X
7	10	–	NTC	27	3	0.01X
8	10	–	NTC	27	3	0.001X

[1] 2 µl of each control and sample dilution will be used in each qRT-PCR reaction
[2] See protocol for additive source.
[3] copies/µl

7. **In your qPCR reaction assembly area,** place a 96-well PCR plate on ice (or a blueblock; 4°C), and dispense 23 µl/well of MRM into the appropriate wells (in duplicate) using a repeating pipet.
8. Using a multichannel pipet, transfer 2 µl/well of the control dilutions, NTCs, and sample dilutions (in duplicate) from the 8-well PCR strips to the PCR plate containing MRM.
9. Centrifuge the plate at 2,000 rpm (4°C) for 2 min to remove any bubbles.

10. Program your real-time qPCR instrument for the following qRT-PCR reaction cycles. Include a final dissociation curve cycle. Install the plate in the instrument and start the program.

    - **RT Reaction**
      - 42°C 5 min
      - 95°C 10 sec
    - **qPCR x 40 Cycles**
      - 95°C 5 sec
      - 60°C 30 sec
    - **Dissociation Curve**
      - 95°C 15 sec
      - 60°C 30 sec
      - All (60°C–95°C)

D. Data Analysis

1. Determine average Ct values from the control dilution duplicates and plot vs. copy number (log scale) to generate a standard curve (Figure 2).
2. Determine average Ct values for each duplicate sample dilution and read the corresponding copy number value from the standard curve. Use all Ct values that are below that of the NTC.
3. For each dilution, back-calculate a starting copy number value for the original sample using the example given below. Generate a mean value to determine the RNA genome content of the sample.

ATTENTION: Be sure to include all dilution steps in the calculation, i.e. the DNase I treatment results in a 2-fold dilution.

Calculating RNA copy numbers and infectivity coefficients, an example:

1. **Copy numbers:** 150 µl of a sample was purified and eluted in 50 µl. The undiluted sample corresponded to a raw copy number of 1 x 10^7 copies on the qRT-PCR Standard Curve.
   - $\text{Copies/ml} = \frac{(1 \times 10^7 \text{ copies})(1{,}000 \text{ µl/ml})(2\text{X DNase})(50 \text{ µl elution*})}{(150 \text{ µl sample*})(2 \text{ µl added to well})}$
   - Copies/ml = 3.33 x 10^9

   *These values are user defined.

NOTE: NTC values average ~35 Ct in our experiments.

2. **Infectivity coefficients:** If you have also determined viral infectivity (i.e. via FACS), calculate an RT:FACS ratio (copies/IFU), or infectivity coefficient, for your virus by dividing the qRT-PCR copies/ml by the IFU/ml value from your FACS titration. This coefficient can then be used to calculate the IFU/ml for subsequent qRT-PCR titration results.
   - Using the copy number from the above example (3.33 x 10^9) and an RT:FACS ratio from Table I, "584", IFU/ml = (3.33 x 10^9 copies/ml)/(584 copies/IFU)
   - IFU/ml = 5.7 x 10^6

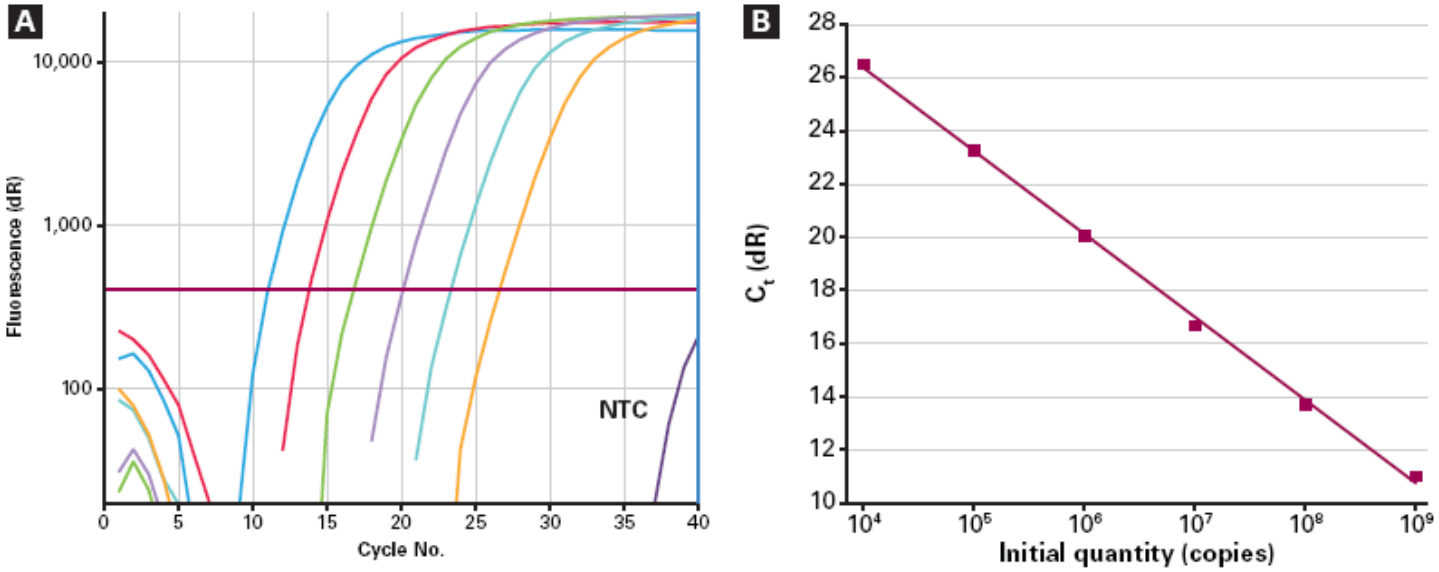

Figure 2. Using the Retro-X RNA Control Template to generate a standard curve. **Panel A.** Amplification plots of a qRT-PCR reactions using serial dilutions of the Retro-X RNA Control Template (10^9–10^4 copies) and the Retro-X qRT-PCR Titration Kit. The assay shows a dynamic range of at least six orders of magnitude with low No Template-Control background (not shown). **Panel B.** A standard curve created from the plots shown in Panel A demonstrates a strong linear correlation between the Ct values and the RNA copy numbers (log scale), with $R^2 = 0.999$ and a PCR efficiency of 108.9%.

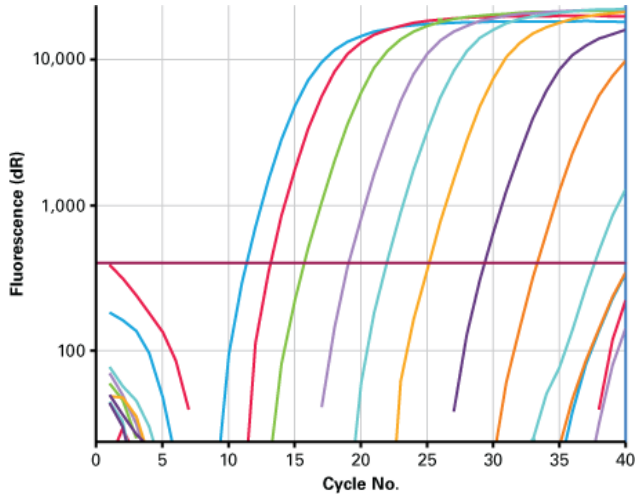

Figure 3. Titration of a retroviral supernatant using the Retro-X qRT-PCR Titration Kit. Representative results from a titration using tenfold serial dilutions of purified retroviral RNA. Amphotrophic retrovirus was produced by transient transfection using Takara Bio's Retro-X Universal Packaging System. Three days posttransfection, retroviral supernatant was collected and stored at –80°C prior to RNA purification and qRT-PCR titration.

V. Troubleshooting Guide

Table V. Troubleshooting Guide for Retro-X qRT-PCR Titration

Problem	Possible Explanation	Solution
High signal in NTC reactions	Contamination of buffer, pipets, or work area from improper handling of samples or control template	Diagnosis: Run control and NTC qRT-PCR sample on 3% agarose gel to visually compare size of product bands (MMLV-specific vs. nonspecific) and compare dissociation curves. Specific amplimer used for titration is ~140 bp. Dissociation curves should reflect the presence of a single product of this size, which should also be visible in the agarose gel.
		Prepare work area properly and use clean, dedicated pipets for each phase of the protocol: dilution, reaction set-up, and analysis.
Poor efficiency or R^2	Poor technique or inconsistent pipetting	Review qRT-PCR techniques; use repeating pipets and multichannel pipets for improved accuracy; calibrate pipets.
Viral signal is higher than expected	High virus yield or residual plasmid DNA contamination due to incomplete DNase I digestion	Repeat DNase I treatment or include it, if omitted. Ensure that samples are free of contaminating plasmid DNA. Perform a control reaction without RT.
Viral signal absent or lower than expected	Purified viral RNA samples contaminated with RNase. DNase contamination in PCR reactions.	Review techniques and condition of work area; include RNase inhibitor in samples, reactions.

VI. References

Carmo, M. *et al.* Quantitation of MLV-based retroviral vectors using real-time RT-PCR. *J. Virol. Methods* **119,** 115–119 (2004).

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