Retro-X™ qRT-PCR Titration Kit Protocol-At-A-Glance (PT3952-2)

THIS PROTOCOL-AT-A-GLANCE IS NOT INTENDED FOR FIRST-TIME USERS.
If you have not used this kit before, please refer to the User Manual (PT3952-1).

I. Protocol: Purifying Retroviral Genomic RNA

1. Use the NucleoSpin RNA Virus Kit to purify RNA from **150 µl** of harvested retroviral supernatant; smaller volumes (5–100 µl) can be used if necessary. Consult the User Manual for buffer preparation instructions.

   NOTE: Omit the initial 70°C incubation of the sample in the kit's protocol. The 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 the step can be omitted in 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.

2. Elute the RNA in 50–100 µl RNase-free water.
3. When titrating retrovirus from stable packaging cell lines, proceed directly to qRT-PCR in Protocol B. When titrating retrovirus from transiently transfected packaging cell lines, first treat the RNA sample(s) with a DNase I reaction as shown in Table 1:

Table 1: 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

1 An RNase inhibitor may be included in the DNase I reaction but is generally unnecessary.

Combine reagents, mix, and incubate first at 37°C for 30 min, and then at 70°C for 5 min. Store the tubes on ice until ready to perform qRT-PCR (Section II).

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

1. On ice, assemble enough Master Reaction Mix (MRM), plus ~10% extra, to perform each reaction in duplicate (Table 2). The MRM should consist of the following reagents (add the RT Enzyme Mix last):

Table 2: 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!

2. In 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). Add EASY Dilution Buffer to the appropriate wells of the strips, as shown in Table III. EASY Dilution Buffer alone in wells 6–8 of Strip 1 will be used for the no-template controls (NTC).
3. 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.
4. Serially dilute your viral RNA samples as shown in Table 3. 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.
5. Mix the strips well by tapping gently, and centrifuge the strips at 2000 rpm (4°C) for 1 min to remove any bubbles.

Table 3: 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

6. Use a repeating pipet to dispense 23 µl of MRM into the appropriate wells of a 96-well PCR plate on ice.
7. Using a multichannel pipet, transfer 2 µl of the control dilutions, NTCs, and sample dilutions from the 8-well strips to duplicate PCR plate wells containing MRM.
8. Centrifuge the plate at 2000 rpm (4°C) for 2 min to remove any bubbles.
9. 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)

III. Data Analysis

Refer to User Manual.

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