Takara Bio USA, Inc.

Adeno-X™ qPCR Titration Kit User Manual

Cat. No. 632252
(050819)

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I. Introduction

A. Summary

The Adeno-X qPCR Titration Kit provides a fast and simple method for titrating adenoviral stocks. The kit employs a quick DNA purification step and determines viral DNA genome content using qPCR and TB Green® chemistry. Titration can be completed in only 4 hours and is designed for use with all Ad5-based vectors, including recombinant adenovirus created with our Adeno-X Expression Systems. Using this kit reduces time delays between virus harvest and target cell infection, allowing you to do both on the same day.

B. Protocol Overview

Adenovirus can be quantitated from either crude adenoviral lysate or purified viral particles. First, a small aliquot of lysate or purified virus is treated with DNase I to remove any residual host cell DNA or plasmid DNA that may have been carried over from the packaging cells. The prep is then treated with Proteinase K to remove the DNaseI and open up the viral particle, and the included viral DNA purification kit is used to purify the viral genomic DNA. Next, serial dilutions of the viral DNA sample are subjected to qPCR to determine the threshold cycle (Ct) for each dilution. The DNA copy number in a diluted sample is then determined from a standard curve generated by plotting the Ct values of the diluted Adeno-X DNA Control Template against their respective copy numbers.

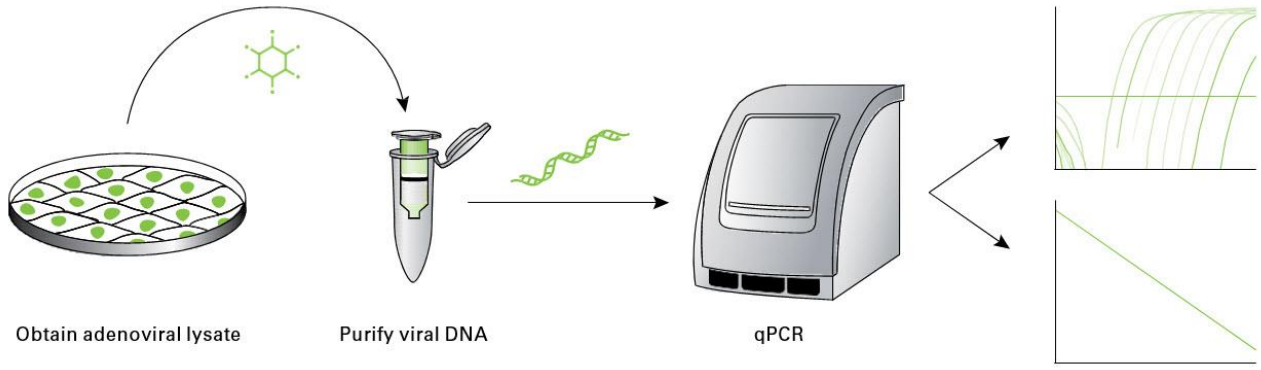

Figure 1. Flowchart of the procedure for titrating adenovirus with the Adeno-X qPCR Titration Kit.

C. Correlating Viral Titer with Infectivity

Once the genome copy number of your viral stock is determined, it can be correlated with the number of viral infectious units (IFU; determined independently) to establish an infectivity coefficient (copy number/IFU; see Table V). Determination of the infectivity coefficient for a given prep allows you to normalize the amount of prep used in each experiment, for consistent inter-assay results. Representative infectivity coefficients (determined with different infectivity titration methods) for a typical Adeno-X virus are shown in Table V. These values should be consistent for similarly prepared viral stocks. However, the calculated ratio may vary due to differences in the amount of virus obtained from individual adenoviral amplifications. Variations in the amount of virus amplified can be caused by differences in cell number, inoculum amount, or time elapsed before the cytopathic effect is observed; therefore, a standardized amplification procedure should be used to help ensure consistent results.

II. List of Components

Store the Adeno-X qPCR Titration Kit Components at –20°C.
Store the TB Green Advantage® qPCR Premix and ROX Reference Dyes (Cat. No. 636976) at –70°C in the dark. After thawing, store at 4°C in the dark. Do not refreeze.
Store the NucleoSpin Virus kit at room temperature.

Adeno-X qPCR Titration Kit (200 rxns; Cat. No. 632252)

Adeno-X qPCR Titration Kit components (not sold separately)

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

TB Green Advantage qPCR Premix (200 rxns; Cat. No. 639676)

- 4 tubes 2X TB Green Advantage qPCR Premix (0.625 ml per tube)
- 100 µl 50X ROX Reference Dye LSR
- 100 µl 50X ROX Reference Dye LMP

NucleoSpin Virus (10 preps; Cat. No. 740983.10)

- 13 ml Lysis Buffer VL
- 6 ml Wash Buffer VW1
- 6 ml Wash Buffer VW2 (Concentrate)
- 13 ml RNase-free H_2O
- 300 µg Carrier RNA (lyophilized)
- 120 µl Liquid Proteinase K
- 20 ml Collection tubes (1.5 ml) for lysis and elution
- 10 NucleoSpin Virus Columns (light red rings, plus Collection Tubes)
- 30 Collection Tubes (2 ml)

III. Additional Materials Required

- Work areas and pipettors free of contaminating DNA and DNases.
- Quantitative real-time PCR thermal cycler (e.g., Mx3000P, Stratagene; ABI 7900, Applied Biosystems; or equivalent)
- Ethanol
- PCR-grade water
- 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. Adenoviral Titration Protocols

Please read these Protocols in their entirety before starting successful titration results depend on performing the following steps in sequence.

A. General Recommendations

Due to the tremendous amplification power and sensitivity of qPCR, even trace amounts of contaminating DNA will be amplified and will affect Ct and final copy number values. Before you begin, prepare work areas free of potentially contaminating DNA and DNases. If possible, dilute your samples and controls in one work area with a dedicated set of pipettors, and assemble your qPCR 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. Preparation of NucleoSpin Virus Kit Buffers

Important: Lysis Buffer VL and Wash Buffer VW1 contain guanidine salts! Wear gloves and goggles!

Storage:
All kit components can be stored at room temperature (20–25°C) and are stable for up to one year.

Liquid Proteinase K:
Liquid Proteinase K is ready to use. After first use, store Liquid Proteinase K at 4°C or –20°C.

Wash Buffer VW2 (Concentrate):
Before using the kit for the first time, add 24 ml ethanol (96–100%; nondenatured ethanol is recommended) to Wash Buffer VW2 (Concentrate). Mark the label of the bottle to indicate that the ethanol is added. Store Wash Buffer VW2 at room temperature (18–25°C).

Carrier RNA:
Carrier RNA (300 µg) is delivered in lyophilized form. Dissolve Carrier RNA in RNase-free water to obtain a stock solution (1 µg/µl). Store Carrier RNA stock solution at –20 °C. Due to the production procedure and the small amount of Carrier RNA contained in the vial, the Carrier RNA may hardly be visible in the vial.

C. Protocol: Purifying Adenoviral Genomic DNA

1. Treat 200 µl of crude, clarified lysate or purified adenoviral particles with DNase I as indicated in Table I. Smaller volumes (50–100 µl) of sample can be used; however, it is necessary to bring the volume up to 200 µl with medium or PBS.

Table I. DNAse I reaction

Reagent	Volume (µl)
Adenoviral Sample	200.0
DNase I (5 units/µl)	5.0
Total	**205.0**

   Combine the reagents, mix, and incubate in a thermal cycler or heat block at 37°C for 30 min.

2. Add 5 µl Liquid Proteinase K to the DNase I-treated sample. Pipette up and down, then add 200 µl Lysis Buffer VL to the tube and vortex for 10–15 sec.

3. If the resulting solution is still turbid, centrifuge the mixture for 1 min at 11,000*g* and collect the supernatant in a sterile centrifuge tube.

4. Add 5.6 µl Carrier RNA stock solution (1 µg/µl) to the sample and mix by vortexing (10–15 sec). Incubate sample for 3 min at room temperature. If necessary, briefly spin the tube to collect liquid in bottom of tube (~1 sec at 2,000*g*).

5. Add 200 µl ethanol (96–100 %) to the tube and mix by vortexing (10–15 sec). Incubate for 5 min at room temperature (18–25°C). Briefly centrifuge the collection Tube (~1 sec at ~ 2,000*g*) to remove drops from the lid (short spin only). Do not centrifuge at a higher g-force in this step!
6. For each sample, place one NucleoSpin Virus column in a 2 ml collection tube and load 610 µl of the sample. Centrifuge for 3 min at 4,000*g*.

   NOTE: If the lysate is not completely drawn through the membrane, repeat the centrifugation at higher g-forces (15,000–20,800*g* for 1 min). In case the lysate still does not pass the membrane completely, discard the sample and repeat the isolation with new sample material.
7. Place the NucleoSpin Virus Column into a new Collection Tube (2 ml, provided) and discard the Collection Tube with flow-through from the previous step.
8. Wash and dry the silica membrane as follows:
   a. Add 400 µl Wash Buffer VW1 to the NucleoSpin Virus Column. Centrifuge for 30 sec at 11,000*g*. Place the NucleoSpin Virus Column into a new Collection Tube (2 ml, provided) and discard the Collection Tube with flow-through from the previous step.
   b. Add 400 µl Wash Buffer VW2 to the NucleoSpin Virus Column. Centrifuge for 30 sec at 11,000*g*. Place the NucleoSpin Virus Column into a new collection tube (2 ml, provided) and discard the Collection Tube with flow-through from the previous step.

      NOTE: Make sure that residual buffer from the previous step is washed away with Wash Buffer VW2, especially if the lysate has been in contact with the inner rim of the column during loading of the lysate onto the column. For efficient washing of the inner rim, flush it with Wash Buffer VW2.
   b. Add 200 µl Wash Buffer VW2 to the NucleoSpin Virus Column. Centrifuge for 5 min at 20,000*g* (or full speed). Place the NucleoSpin Virus Column in a clean Elution Tube (1.5 ml, provided) and discard the Collection Tube with flow-through from the previous step. Incubate the assembly for 5 min at 56 °C with open column lid.
9. To elute the DNA, add 30 µl RNase-free H_2O (preheated to 70°C) onto the column. Incubate for 3 min at room temperature. Centrifuge 3 min at 20,000*g* to elute nucleic acid from the column.
10. Keep eluted DNA on ice or freeze for storage. Perform qPCR (Section D).

D. Protocol: qPCR Amplification of Adenoviral Genomic DNA

1. **In your reaction assembly work area,** make a Master Reaction Mix (MRM) on ice consisting of the reagents in Table II. Make sufficient MRM for the required number of wells. Each control, no-template control (NTC), and sample reaction should be performed in duplicate:

Table II. Master Reaction Mixes Recommended for Different qPCR Instruments

Reagent	qPCR Instrument			
	Stratagene Mx3000P	Takara Bio Thermal Cycler Dice Real Time System	Applied Biosystems Instruments	Roche LightCycler
	Reagent volume (µl per well) for each instrument			
PCR-grade H_2O	9.0	9.5	6.8	7.2
Adeno-X Forward Primer (10 µM)	0.5	0.5	0.4	0.4
Adeno-X Reverse Primer (10 µM)	0.5	0.5	0.4	0.4
ROX™ Reference Dye LSR **or** LMP (50X)*	0.5	—	0.4	—
TB Green Advantage qPCR Premix (2X)	12.5	12.5	10.0	10.0
Total volume per well	**23.0**	**23.0**	**18.0**	**18.0**

*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!**

NOTE: To ensure sufficient volume, prepare approximately 10% more Master Reaction Mix than you think you'll need (see example, below).

Example calculation:

Calculating Total Master Reaction Mix (MRM) Volume:

Total MRM Volume = 1.10 x [total number of wells] x [total volume per well]

1. **Controls:** 5 dilutions in duplicate; 1.10 x 10 wells x 23 µl = 253 µl
2. **NTCs:** 3 each in duplicate; 1.10 x 6 wells x 23 µl = 152 µl
3. **Samples:** 4 dilutions in duplicate in duplicate; 1.10 x 8 wells x 23 µl = 202 µl

2. **In your sample dilution work area,** and using PCR grade 8-well strips, dilute the Adeno-X DNA Control Template and purified sample(s) with EASY Dilution Buffer as shown in Table III.

Table III. Control and Sample Dilutions for qPCR

Well	Strips 1 & 2: Controls*	Strip 3, etc.: Samples
1	5×10^7	Sample 1 (1x)
2	5×10^6	0.1x
3	5×10^5	0.01x
4	5×10^4	0.001x
5	5×10^3	Sample 2 (1x)
6	NTC	0.1x
7	NTC	0.01x
8	NTC	0.001x

*copies/µl

a. Dilute the Adeno-X DNA Control Template in an 8-well strip (Table III, 'Strip 1') as follows:
   i. In the first well, pipet 2 µl of the Adeno-X DNA Control Template stock (5 x 10^8 copies/µl) into 18 µl of buffer for a 1:10 dilution (diluted sample = 5 x 10^7 copies/µl).
   ii. Subsequent dilutions (wells 2–5) can be made by serially transferring 3 µl of the preceding dilution into 27 µl of buffer in the next well.
b. Make a duplicate series of dilutions in a second 8-well strip (Table III, 'Strip 2').
c. Pipet only EASY Dilution Buffer into the last 3 wells of both strips for NTC controls.
d. Dilute your DNA sample(s) in another set of 8-well strips; each strip can be used to dilute either 2 duplicate samples or 2 different samples (Table III, 'Strip 3, etc.'). **We recommend making duplicate dilutions of all samples.**
   i. The first well in each series (wells 1 & 5) should contain 20 µl of undiluted sample (1X).
   ii. Subsequent 10-fold sample dilutions (wells 2–4 & 6–8) can be made by serially transferring 3 µl of sample from one well into 27 µl of buffer in the next well.
e. Centrifuge the strips at 2000 rpm (4°C) for 1 min to remove any bubbles.

3. **In your qPCR reaction assembly area,** place a 96-well PCR plate on ice (or on a blueblock; 4°C), and dispense the appropriate total volume of MRM/well for your thermal cycler (e.g., 23 µl/well for Stratagene's Mx3000P, see Table II) into the appropriate wells (in duplicate) using a repeating pipettor.
4. Using a multichannel pipettor, 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.
5. We recommend that you program your real-time qPCR instrument for the following qPCR reaction cycles (see Table IV). Include a final dissociation curve cycle.

Table IV. Recommended Thermal Cycling Conditions for Different qPCR Instruments

Reaction Cycles	qPCR Instrument				
	Stratagene Mx3000P	Takara Bio Thermal Cycler Dice Real Time System	ABI7500 Fast	ABI7000	Roche LightCycler
	Thermal cycling conditions for each instrument				
Denaturation (1 cycle)	95°C 10 sec	95°C 30 sec	95°C 30 sec	95°C 30 sec	95°C 20 sec
qPCR (40 cycles)	95°C 5 sec 60°C 20 sec	95°C 5 sec 60°C 30 sec	95°C 3 sec 60°C 25 sec	95°C 3 sec 60°C 31 secF	95°C 5 sec 60°C 20 sec
Melting/ Dissociation Curve (1 cycle)	95°C 1.5 min 55°C 30 sec 95°C 30 sec	95°C 15 sec 60°C 30 sec 95°C 15 sec	95°C 15 sec 60°C 1 min 95°C 15 sec	95°C 15 sec 60°C 1 min 95°C 15 sec	95°C 0 sec[a] 60°C 15 sec 95°C 0 sec[b]

[a]20°C/sec
[b]0.1°C/sec

NOTE: Although Table IV shows the optimized cycling conditions for a selection of commonly used instruments, the Adeno-X qPCR Titration Kit can be used with a variety of real-time instruments and is not limited to those listed in the table. For instruments not listed, please refer to the TB Green qPCR Premix User Manual (PT3883-1) and/or your instrument's user manual to determine cycling conditions for your particular thermal cycler.

E. 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 the copy number of the original sample (see the sample calculation below). To obtain the viral genome content of the sample, simply calculate the mean copy number.

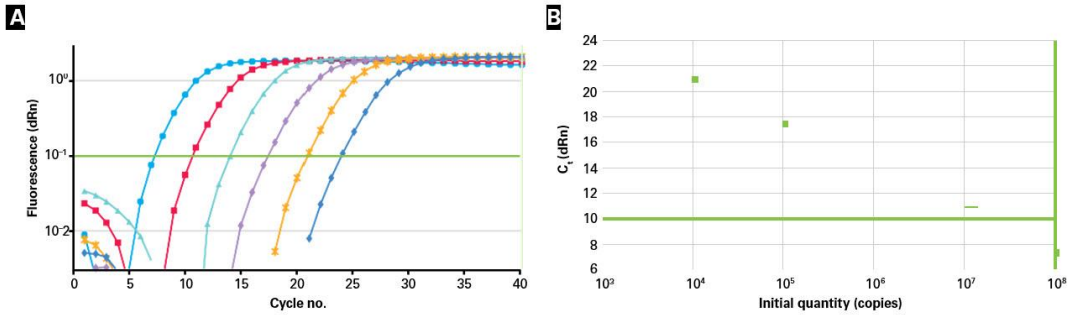

Figure 2. Using the Adeno-X DNA Control Template to generate a standard curve. Panel A. Amplification plots of qPCR reactions using serial dilutions of the Adeno-X DNA Control Template (10^8–10^3 copies) and the Adeno-X qPCR Titration Kit (each dilution is represented by a different colored plot). The assay shows a dynamic range of at least six orders of magnitude. Panel B. A standard curve created from the plots shown in Panel A demonstrates a strong linear correlation between the Ct values and the DNA copy numbers (log scale), with R2 = 1.00 and a PCR efficiency of 96.2%.

Sample calculations
Calculating DNA copy numbers and infectivity coefficients:

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^{10} copies on the qPCR standard curve.

Copies/ml = $\frac{(1 \times 10^{10} \text{ copies})(1000 \text{ µl/ml})(50 \text{ µl elution*})}{(150 \text{ µl sample*})(2 \text{ µl per well})}$

Copies/ml = 1.67 x 10^{12}

*These values are user defined.
NOTE: NTC values average ~35 Ct in our experiments

2. Infectivity coefficients: If you have determined the infectivity titer of your virus (e.g., via fluorescence), you can also calculate the infectivity coefficient (i.e., the copy number:infectivity ratio; copies/IFU) for your viral prep. This value is determined by dividing the viral genome copy number (copies/ml; determined by qPCR) by the infectivity titer (IFU/ml; determined

by the infectivity titration method of your choice; see Table V on the next page). Knowing the infectivity coefficient for a given prep allows you to normalize the amount of virus used in each experiment, for consistent inter-assay results.

Table V. Adeno-X qPCR Titration—Correlation of Viral Titer and Infectivity for Crude Lysates and Purified Viral Particles

Sample Type	Virus	Titration Method			Infectivity Coefficients	
		qPCR[a] (copies/ml)	Fluor[b] (IFU/ml)	X-Gal (IFU/ml)	qPCR/Fluor (copies/IFU)	qPCR/X-Gal (copies/IFU)
Purified	AdAcGFP1	5.62×10^9	3.03×10^9	N/A	2	N/A
Crude	AdAcGFP1					
	Prep A	1.44×10^{10}	1.90×10^9	N/A	8	N/A
	Prep B	1.46×10^{10}	2.26×10^9	N/A	6	N/A
	Prep C	1.38×10^{10}	2.73×10^9	N/A	5	N/A
Purified	AdLacZ	1.01×10^{10}	N/A	1.54×10^9	N/A	7
Crude	AdLacZ					
	Prep A	1.33×10^{10}	N/A	2.67×10^9	N/A	5
	Prep B	1.24×10^{10}	N/A	2.67×10^9	N/A	5
	Prep C	1.21×10^{10}	N/A	3.15×10^9	N/A	4

[a] Adenoviral copy numbers were determined using the Adeno-X qPCR Titration Kit (Cat. No. 632252).
[b] To determine fluorescence-based infectivity titers, adenoviral stocks were serially diluted (tenfold) and applied to HEK 293 cells. After 48 hr, fluorescent cells were scored using a fluorescence microscope; LacZ positive cells were scored under phase microscopy.

NOTE: The data shown in Table V are intended for illustrative purposes only. Users should determine infectivity coefficients that are specific for their viral preparation.

Appendix A. Troubleshooting Guide

Table VI. Troubleshooting Guide for Adeno-X qPCR Titration

Description of Problem	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 qPCR sample on 3% agarose gel to visually compare size of product bands (adenoviral-specific vs. nonspecific) and compare dissociation curves. Specific amplimer used for titration is 167 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 pipetting inconsistent	Review qPCR techniques; use repeating pipettors and multichannel pipettors 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 samples are free of contaminating plasmid DNA. Perform a control reaction.
Viral signal absent or lower than expected	Low titer sample	Reamplify virus or concentrate viral stock and retitrate.
	Purified viral DNA samples contaminated with DNase. DNase contamination in PCR reactions.	Review techniques and condition of work area. Treat viral particles with DNase I **prior to** DNA purification

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