User Manual

Adeno-X™ qPCR Titration Kit User Manual

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I. Introduction

A. Summary

The **Adeno-X qPCR Titration Kit** provides a fast and simple method for titrating adenoviral stocks (*Clontechniques,* April 2009). The kit employs a quick DNA purification step and determines viral DNA genome content using qPCR and SYBR® 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 DNase I 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 (C_t) for each dilution. The DNA copy number in a diluted sample is then determined from a standard curve generated by plotting the C_t values of the diluted Adeno-X DNA Control Template against their respective copy numbers.

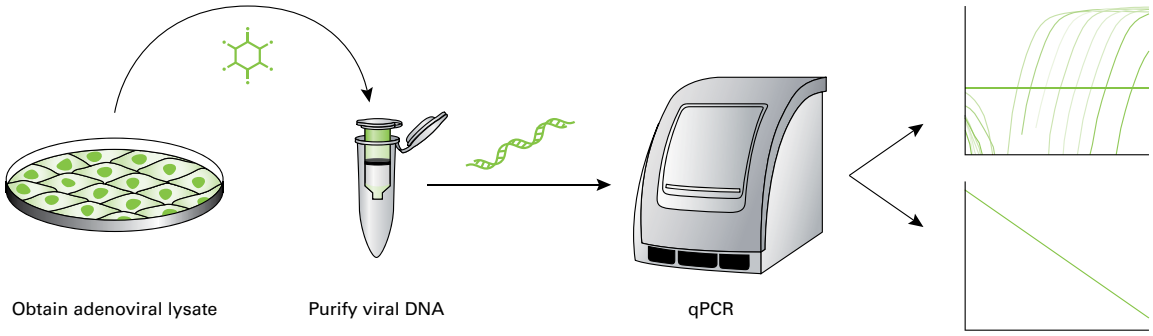

Figure 1. Flowchart of the proceedure for titrating adenovirus with the Adeno-X qPCR Titration Kit

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, pg. 11). Determination of the infectivity coefficient for a given prep allows you to normalize the amount of prep used in each experiment, for consistent interassay 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, innoculum 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 SYBR® 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)

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

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

NucleoSpin Virus Kit (10 preps; Cat. No. 740977.10)

- 10 ml Lysis Buffer RAV1
- 6 ml Wash Buffer RAW
- 12.5 ml Wash Buffer RAV3(Concentrate)
- 5 ml RNase-free H_2O
- 5 ml Elution Buffer RE (5 mM Tris/HCl, pH 8.5)
- 300 µg Carrier RNA (lyophilized)
- 6 mg Proteinase K (lyophilized)
- 0.8 ml Proteinase Buffer PB
- 10 NucleoSpin Virus Columns (dark blue 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 C_t 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: RAV1 and RAW Buffers 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.
- **Proteinase K:**
  Before using the kit for the first time, add 260 µl Proteinase Buffer to dissolve the lyophilized Proteinase K. Proteinase K solution is stable at –20°C for 6 months.
- **RAV1 Buffer:**
  1. Before using the kit for the first time, add 1 ml RAV1 Buffer to the vial containing Carrier RNA. Dissolve the RNA and transfer it back to the RAV1 bottle.
  2. Carrier RNA has a limited shelf life in RAV1 Buffer. RAV1 Buffer containing Carrier RNA:
     a. Can be stored at room temperature for 1–2 weeks. Room temperature storage prevents salt precipitation.
     b. Can be stored at 4°C for up to 4 weeks or aliquoted and stored at –20°C for longer periods. Storage at or below 4°C may cause salt precipitation; therefore, to redissolve the salt, the mixture must be preheated at 40–60°C for a maximum of 5 min.

Note: **Do not warm RAV1 Buffer containing Carrier RNA more than 4 times!** Frequent warming, temperatures > 80°C, and extended heat incubation will accelerate the degradation of Carrier RNA. This leads to reduced recovery of viral DNA and eventually false negative qPCR results, particularly if low-titer samples are used.

- **RAV3 Buffer:**
  Add 50 ml ethanol (96–100%) to the bottle of RAV3 Buffer. Mark the label of the bottle to indicate that the ethanol has been added. Store the RAV3 Buffer at room temperature (20–25°C) for up to one year.

IV. Adenoviral Titration Protocols continued

C. Protocol: Purifying Adenoviral Genomic DNA

Protocol
1 hr

1. Treat 150 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 150 µl with medium or PBS.

Table I: DNase I Reaction

Reagent	Volume (µl)
Adenoviral Sample	150.0
DNase I (5 units/µl)	5.0
Total	**155.0**

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

2. Add 600 µl RAV1 Buffer (containing Carrier RNA) and 20 µl Proteinase K (20 mg/ml) to the DNase I-treated sample. Pipette up and down, then vortex for 10–15 sec and incubate the sample at 70°C for 5 min.
3. If the resulting solution is still turbid, centrifuge the mixture for 1 min at 11,000 x g and collect the supernatant in a sterile centrifuge tube.
4. Add 600 µl ethanol (96–100%) to the Proteinase K-treated sample and mix by vortexing (10–15 sec).
5. For each sample, place one NucleoSpin Virus column in a 2 ml collection tube and load 700 µl of the sample. Centrifuge for 1 min at 8,000 x g.

   Note: When preparing infectious material, use new collection tubes for every step. This decreases the chance of cross-contamination and contamination of the centrifuge. For non-infectious samples, discard the flow-through and reuse the collection tube for loading and washing steps.

6. Load the remaining sample onto the NucleoSpin column. Centrifuge for 1 min at 8,000 x g. Discard the flow-through and put the NucleoSpin column into a new collection tube. More than two loading steps are not recommended.
7. Wash and dry the silica membrane as follows:
   a. Add 500 µl RAW Buffer to the NucleoSpin column. Centrifuge for 1 min at 8,000 x g, and discard the flow-through.
   b. Add 600 µl RAV3 Buffer to the NucleoSpin column. Centrifuge for 1 min at 8,000 x g, and discard the flow-through and the collection tube.
   c. Place the NucleoSpin column in a new collection tube and add 200 µl RAV3. Buffer. Centrifuge for 2–5 min at 11,000 x g.
8. To elute the DNA, place the NucleoSpin column into a sterile, new 1.5 ml microcentrifuge tube. Add 50 µl sterile H_2O or RE buffer (preheated to 70°C) and incubate for 1–2 min. Centrifuge for 1 min at 11,000 x g.
9. Store the DNA on ice until you're ready to perform qPCR (Section C).

IV. Adenoviral Titration Protocols continued

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

	qPCR Instrument			
	Stratagene Mx3000P	Takara Bio Thermal Cycler Dice Real Time System	Applied Biosystems Instruments	Roche LightCycler
Reagent	**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	
SYBR® 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

IV. Adenoviral Titration Protocols continued

D. Protocol: qPCR Amplification of Adenoviral Genomic DNA continued

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 (5×10^8 copies/µl) into 18 µl of buffer for a 1:10 dilution (diluted sample = 5×10^7 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.

IV. Adenoviral Titration Protocols continued

D. Protocol: qPCR Amplification of Adenoviral Genomic DNA continued

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

	qPCR Instrument									
	Stratagene Mx3000P		Takara Bio Thermal Cycler Dice Real Time System		ABI7500 Fast		ABI7000		Roche LightCycler	
Reaction Cycles	Thermal cycling conditions for each instrument									
Initial 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 60°C	5 sec 20 sec	95°C 60°C	5 sec 30 sec	95°C 60°C	3 sec 25 sec	95°C 60°C	3 sec 31 sec	95°C 60°C	5 sec 20 sec
Dissociation Curve (1 Cycle):	95°C 55°C 95°C	1 min 30 sec 30 sec	95°C 60°C 95°C	15 sec 30 sec 15 sec	95°C 60°C 95°C	15 sec 1 min 15 sec	95°C 60°C 95°C	15 sec 1 min 15 sec	95°C 60°C 95°C	0 sec[a] 15 sec 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 SYBR® qPCR Premix User Manual (PT3883-1) and/or your instrument's user manual to determine cycling conditions for your particular thermal cycler.

IV. Adenoviral Titration Protocols continued

E. Data Analysis

1. Determine average C_t values from the control dilution duplicates and plot vs. copy number (log scale) to generate a standard curve (Figure 2).
2. Determine average C_t values for each duplicate sample dilution and read the corresponding copy number value from the standard curve. Use all C_t 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.

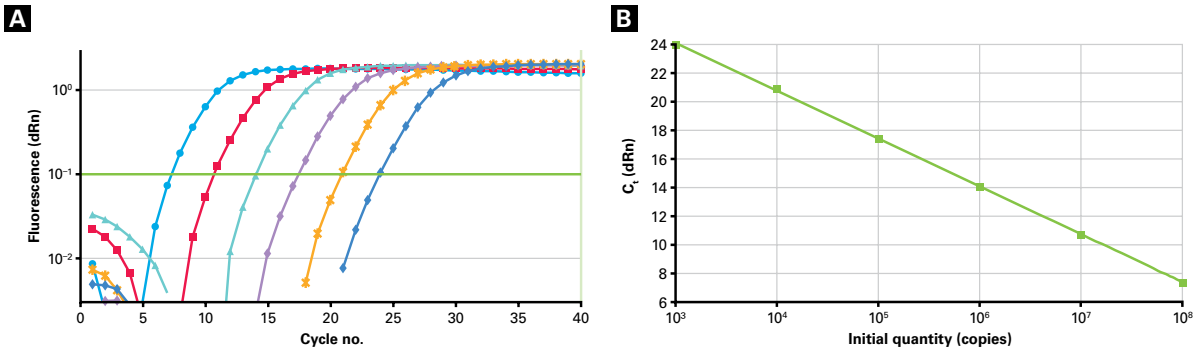

Figure 2. Using the Adeno-X DNA Control Template to generate a standard curve. Panel A. Amplification plots of qPCR reactions using serial dilutions of the Adeno-X DNA Control Template (10^8–10^3 copies) and the Adeno-X qPCR Titration Kit (each dilution is represented by a different colored plot). The 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 C_t values and the DNA copy numbers (log scale), with $R^2 = 1.00$ and a PCR efficiency of 96.2%.

Example

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 = $\frac{(1 \times 10^{10}\ \text{copies})(1000\ \mu\text{l/ml})(50\ \mu\text{l elution*})}{(150\ \mu\text{l sample*})(2\ \mu\text{l per well})}$
   - Copies/ml = 1.67 x 10^{12}

   *These values are user defined.

   Note: NTC values average ~35 C_t 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 coeffcient (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 coeffcient for a given prep allows you to normalize the amount of virus used in each experiment, for consistent interassay results.

IV. Adenoviral Titration Protocols continued

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

		Titration Method			Infectivity Coefficients	
Sample Type	**Virus**	**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.

V. Troubleshooting

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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