Protocol ● AAVPrime™ AAV-qPCR Titration Kits ● Cat. No. AA301 / AA302

Description

The AAV-qPCR Titration Kits are designed for quick and simple titration of Adeno-associated virus (AAV) particles using real-time quantitative PCR (qPCR), it can determines titration of all AAV serotypes as long as the ITR domains of the AAV genome is of AAV2 origin. The kits include the DNase I (RNase-free), AAV Lysis buffer, AAV-TaqMan qPCR mix (2x), AAV-Probe, and standard control. The number of copies of the AAV genome is quantified using TaqMan –based qPCR.

Kit features:

- Confirms the success of AAV particle production
- Determines the virus copy number to ensure the efficiency of cell transduction and gene expression
- Includes reagents needed for all steps in titration, from viral DNA extraction to qPCR
- Quantification is based on amplification of the ITRs (inverted terminal repeats) of AAV
- Determines titration of all AAV serotypes as long as the ITR domains of the AAV genome is of AAV2 origin
- Simplifies AAV genome extraction and generates quick results

Contents and Storage

AA301 (20 DNase reactions, 50 qPCR reactions); AA302 (40 DNase reactions, 100 qPCR reactions).

Component	Volume	Concentration	Shipping	Storage
DNase I (RNase-free)	1x20 µL 2x20 µL	2,000 units/ml	Ice pack	-20°C, Stable for at least 12 months
DNase I buffer (10x)	1x20 µL 2x20 µL		Ice pack	-20°C, Stable for at least 12 months
AAV Lysis buffer	1x200 µL 2x200 µL		Ice pack	-20°C, Stable for at least 12 months
AAV-TaqMan qPCR mix (2x)	1x500 µL 2x500 µL		Ice pack	-20°C, Stable for at least 12 months
AAV-qPCR primer mix	1x100 µL 2x100 µL	2.5 µM	Ice pack	-20°C, Stable for at least 12 months
AAV-Probe	1x100 µL 2x100 µL	2.5 µM	Ice pack	-20°C, Stable for at least 12 months
AAV-qPCR standard (DNA)	1x25 µL 2x25 µL	$1x10^9$ copies/µl	Ice pack	-20°C, Stable for at least 12 months
ddH_2O	1x1 mL 2x1 mL		Ice pack	Room temperature, Stable for at least 12 months

Materials Required but Not Provided

General equipment for cell culture

Real-time PCR machine and tubes

Related Products

HEK293T AAV packaging cell line

Transfection Reagent (e.g.,Endofectin-Lenti Transfection Reagent, GeneCopoeia cat.#EF001)

Protocol

Please read the entire procedure carefully before starting the experiment. The following procedures include the process of producing AAV cells and extracting AAV particles.

1. Production of AAV stocks

1) Prepare a dry ice-ethanol bath and 37°C water bath.

2) For a 10 cm dish containing AAV producing cells (usually 72 hours after transfection of AAV packaging plasmids), scrape the cells using a cell scraper and transfer both the cells and culture medium into a 15 mL centrifuge tube.

3) Subject the cell suspension to four rounds of freeze/thaw by alternating the tubes between the dry ice-ethanol bath and the 37°C water bath, vortexing briefly after each thaw.

4) Remove cellular debris by centrifugation at 10000 x g for 10 min at room temperature.

5) Transfer the supernatant to a fresh tube; this is the crude AAV product.

6) The crude AAV product can be used directly for the titration assay. For testing purified/concentrated AAV samples, several serial dilutions are needed to make sure the AAV titer will fall into the range of the standard curve.

7) For long term storage, store crude or purified AAV products at -80ºC.

2. Extraction of AAV Genome

1) Treat the AAV particles (crude or purified) with DNase I to digest free genomic DNA and plasmid derived from packaging cells.

DNase I digestion (to eliminate AAV expression plasmid contamination). In a 200μL tube set up the following reaction (total volume 10 μL):

Crude AAV or diluted purified AAV*	8 μL
10x DNase I Buffer	1 μL
DNase I	1 μL
Total	10 μL

Then incubate:

37°C for 20 minutes (digest free genomic DNA and plasmid)

95°C for 10 minutes (to inactivate DNase I)

8°C indefinitely

(If doing this on thermocycler, do not use the heat lid function for the first step)

*Purified AAV should be diluted with PBS. The dilution factor should be determined by the concentration factor compared with the crude.

2) Lysis of AAV particles

Add 10 µL AAV Lysis buffer into the product from Step (1), vortex and spin, then incubate

65°C for 30 minutes

95°C for 10 minutes

8°C indefinitely
(If doing this on thermocycler, do not use the heat lid function for the first step)

This will be the lysate sample for the next step of the Real-time qPCR reaction.

To make sure the AAV amount falls into the linear range of the standard curve, make several dilutions of the lysate from step 2-(2) with ddH2O:

A: Original lysate without dilution	8 µL
B: 10x dilute	8 µL
C: 100x dilute	8 µL
D: 1000x dilute	8 µL

3) Real-time qPCR

1) Sample Preparation for Standard Curve

Dilute the positive control to obtain the samples for standard curve preparation. (Use 5 µL of each solution as a template for qPCR.)

(1) 2 x 10^7 copies/µL (5 µL of AAV-qPCR standard (DNA) + 245 µL of ddH2O)

(2) 2 x 10^6 copies/µL (5 µL of (1) + 45 µL of ddH_2O)

(3) 2 x 10^5 copies/µL (5 µL of (2) + 45 µL of ddH_2O)

(4) 2 x 10^4 copies/µL (5 µL of (3) + 45 µL of ddH_2O)

(5) 2 x 10^3 copies/µL (5 µL of (4) + 45 µL of ddH_2O)

(6) 2 x 10^2 copies/µL (5 µL of (5) + 45 µL of ddH_2O)

2) Set up the following qPCR reaction (total volume 20 µL):

AAV-TaqMan qPCR mix (2x)	10 µL
DNA standard or lysate sample	5 µL
Primer Mix (final concentration 0.25 µM each)	2 µL
Probe (final concentration 0.25 µM)	2 µL
ddH_2O	up to 20 µL
Total	20 µL

Notes:

- A master mix that includes all but the DNA standard/sample is recommended for all reactions.
- A no-template control (NTC) should be included.
- Suggested amounts of DNA standard: (See the form as below)

Amounts of DNA standard	1×10^8	1×10^7	1×10^6	1×10^5	1×10^4	1×10^3
Equal to volume from step 1)	5 µL of (1)	5 µL of (2)	5 µL of (3)	5 µL of (4)	5 µL of (5)	5 µL of (6)

3) Start qPCR reaction cycles

The following settings are optimized for the ABI-ViiA 7 real time PCR detection system. You may need to fine tune the cycling conditions for the system you are using.

Cycle	Steps	Temperature	Duration	Read
1	Denaturation	95 °C	10 min	off
35	Denaturation	95°C	10 sec	off
	Annealing	60 °C	20 sec	off
	Extension	72 °C	25 sec	on

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Detection of Fluorescence: FAM
Quencher: BHQ1

4) Data analysis

(1) Determine the Ct values for each of the standard DNAs. Plot the Ct against the copy number (log scale) to generate a standard curve. The correlation coefficient of the standard curve should be above 0.99.

(2) Determine the Ct values for the samples. Retrieve the copy number for the corresponding Ct value, or determine the copy number by using the standard curve generated in the previous step.

(3) Multiply the above raw copy number by the **dilution factor** to get the copy number for the original sample (copies/mL).

$$\text{Dilution factor} = \frac{\text{DNase reaction volume(ul)}}{\text{Original sample volume(ul)}} \times \frac{\text{Lysis reaction volume(ul)}}{\text{Volume of sample use in lysis reaction(ul)}} \times \frac{\text{1000ul/ml}}{\text{Volume added to PCR well(ul)}}$$

Notes:

- DNase reaction volume: 10 µL using the standard protocol.
- Original sample volume: The volume of AAV particles used for DNase reaction. 8 µL using the standard protocol.
- Lysis reaction volume:20 µL using the standard protocol.
- Volume of sample use inlysis reaction:10 µL using the standard protocol.
- Volume added to PCR well:5 µL using the standard protocol.

(4) Each AAV particle contains one copy of positive single-stranded DNA. Therefore, the number of AAV particles per mL corresponds to the copy number.

(5) Example of the standard curve:

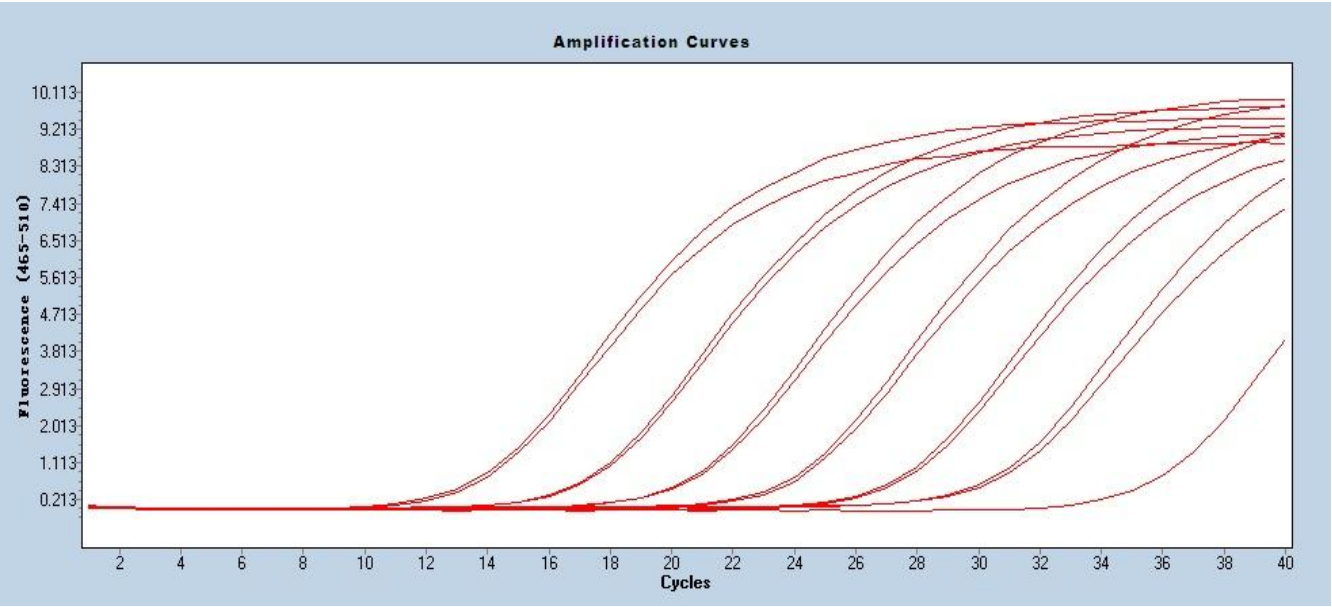

Figure 1. Amplification Plot generated with the AAV-qPCR standard (DNA) and no-template control (NTC).

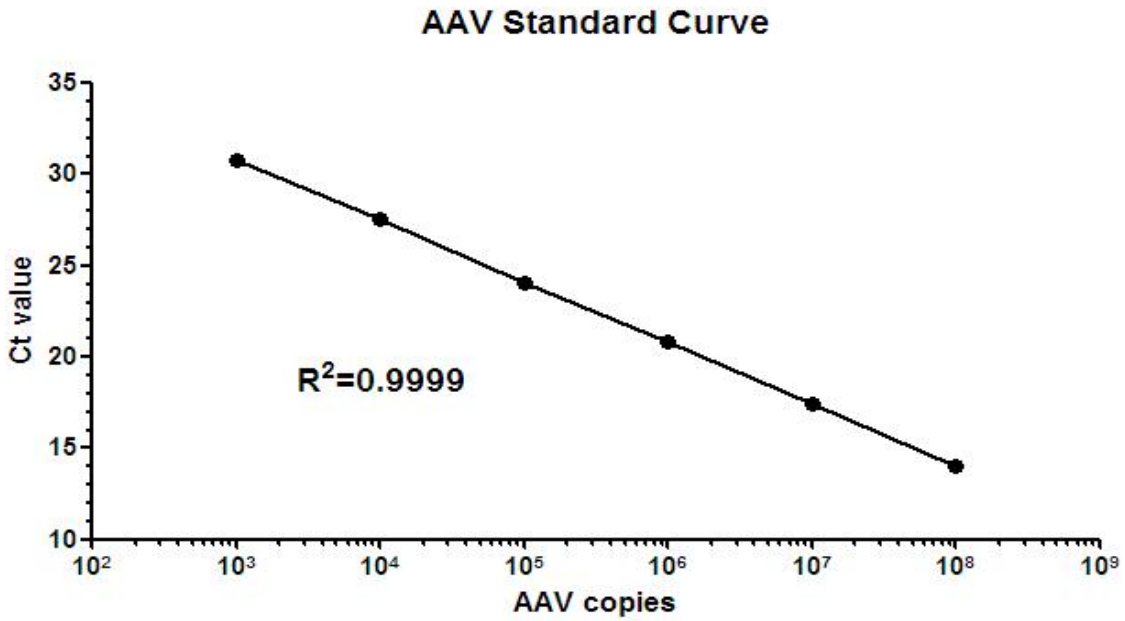

Figure 2. A representative standard curve generated with the AAV-qPCR standard (DNA). Six 10-fold dilution steps ranging from1×10^3 to 1×10^8 copies of the AAV-qPCR standard (DNA) were used to generate the standard curve.

Limited Use License and Warranty

Limited use license

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