Takara Bio USA

AAV-X™ Rapid Titer ELISA Kit (Single Wash) User Manual

Cat. Nos. 631485, 631486, 631487
(031126)

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Table of Contents

I. Introduction ... 3
II. List of Components ... 3
III. Additional Materials Required (Not Provided) ... 4
IV. General Considerations ... 5
V. Protocol: AAV2/8/9 Rapid Titer ELISA Kit (Single Wash) Procedures ... 5
A. Protocol: Prepare Reagents ... 6
B. Protocol: Perform ELISA ... 8
VI. Interpretation of ELISA Reading ... 9
A. Collecting Data ... 9
B. Plotting the Standard Curve ... 9
Appendix A. Troubleshooting Guide ... 11

Table of Figures

Figure 1. Workflow overview for AAV Rapid Titer ELISA Kit (Single Wash) ... 3
Figure 2. Preparation of the AAV stock solution and AAV standard solution ... 7
Figure 3. Preparing the dilution series for the AAV standard curve ... 8
Figure 4. Example standard curves for AAV2, AAV8, and AAV9 ... 10

Table of Tables

Table 1. AAV2 Rapid Titer ELISA Kit (Single Wash) components ... 4
Table 2. AAV8 Rapid Titer ELISA Kit (Single Wash) components ... 4
Table 3. AAV9 Rapid Titer ELISA Kit (Single Wash) components ... 4
Table 4. Example limits of detection (LOD) and limits of quantification (LOQ) ... 10
Table 5. Troubleshooting guide ... 11

I. Introduction

The **AAV-X™ Rapid Titer ELISA Kit (Single Wash) (AAV2)** (Cat. No. 631485), **AAV-X™ Rapid Titer ELISA Kit (Single Wash) (AAV8)** (Cat. No. 631486), and **AAV-X™ Rapid Titer ELISA Kit (Single Wash) (AAV9)** (Cat. No. 631487) provide a streamlined and efficient method for determining AAV (adeno-associated virus) titers by detecting and quantifying AAV capsid proteins of AAV2, AAV8, and AAV9, respectively. The assay utilizes an enzyme-linked immunosorbent assay (ELISA) in a 96-well microtiter plate (12 x 8-well strips). The ELISA plate is pre-coated with anti-AAV capture antibodies that selectively bind the respective AAV capsid proteins (Figure 1). An AAV HRP-conjugated detection antibody (AAV HRP-Detection Antibody) is then added for labeling. After a single wash step, TMB Substrate is added, and absorbance signal intensity is measured. Each kit includes a Lyophilized AAV Empty Particle Control for generating a standard curve. By comparing the signal from the sample to the standard curve, the titer of AAV particles in the sample can be calculated. The kit reliably quantifies titers above 7.81×10^7 VP/mL for AAV2, 2.23×10^7 VP/mL for AAV8, and 2.60×10^7 VP/mL for AAV9.

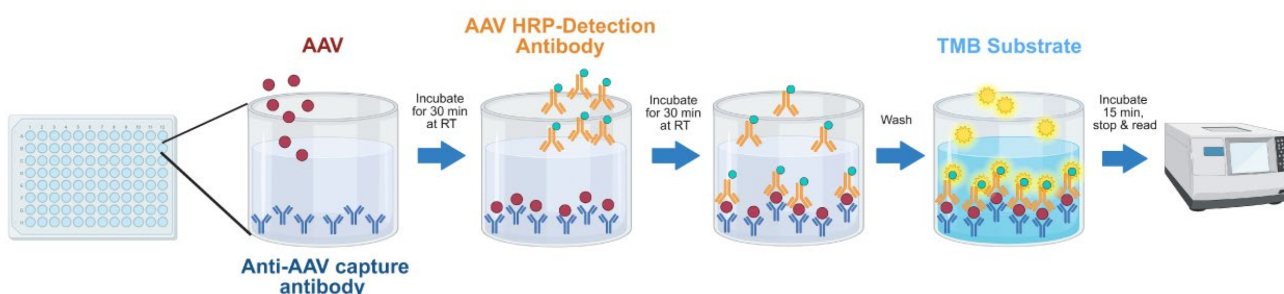

Figure 1. Workflow overview for AAV-X Rapid Titer ELISA Kit (Single Wash) (AAV2/8/9). Anti-AAV capture antibodies, pre-coated on the bottom of the ELISA plate, bind AAV capsid proteins contained in the sample or AAV standard curve dilutions during a 30 min incubation at room temperature (RT). An AAV HRP-conjugated detection antibody (AAV HRP-Detection Antibody) is then added to the plate, followed by another 30 min incubation at RT. After a single wash, TMB Substrate is added and incubated for 15 min. The reaction is stopped with Stop Solution, and the signal is read. AAV quantitation is achieved by comparing sample absorbance values to a standard curve.

II. List of Components

This AAV-X Rapid Titer ELISA Kit (Single Wash) (AAV2/8/9) workflow applies to the following kits:

- AAV-X Rapid Titer ELISA Kit (Single Wash) (AAV2) (Cat. No. 631485)
- AAV-X Rapid Titer ELISA Kit (Single Wash) (AAV8) (Cat. No. 631486)
- AAV-X Rapid Titer ELISA Kit (Single Wash) (AAV9) (Cat. No. 631487)

Table 1. AAV-X Rapid Titer ELISA Kit (Single Wash) (AAV2) components.

AAV-X Rapid Titer ELISA Kit (Single Wash) (AAV2) (Store at 4°C)	631485 (96 rxns)
AAV-X Rapid Titer ELISA Components (Cat. No. 631481)*	
Anti-AAV Coated Plate (96 wells)	1 each
AAV HRP-Detection Antibody	12 ml
Washing Buffer (20X)	50 ml
TMB Substrate	12 ml
Stop Solution	12 ml
AAV-X Rapid Titer ELISA Control (AAV2) (Cat. No. 631482)*	
Lyophilized AAV2 Empty Particle Control	2 x 1xE10 VP
Reconstitution Buffer	2 x 1.5 ml
Diluent Buffer	2 x 20 ml

*Not sold separately.

Table 2. AAV8 Rapid Titer ELISA Kit (Single Wash) components.

AAV-X Rapid Titer ELISA Kit (Single Wash) (AAV8) (Store at 4°C)	631486 (96 rxns)
AAV-X Rapid Titer ELISA Components (Cat. No. 631481)*	
Anti-AAV Coated Plate (96 wells)	1 each
AAV HRP-Detection Antibody	12 ml
Washing Buffer (20X)	50 ml
TMB Substrate	12 ml
Stop Solution	12 ml
AAV-X Rapid Titer ELISA Control (AAV-8) (Cat. No. 631483)*	
Lyophilized AAV8 Empty Particle Control	2 x 1xE10 VP
Reconstitution Buffer	2 x 1.5 ml
Diluent Buffer	2 x 20 ml

*Not sold separately.

Table 3. AAV-X Rapid Titer ELISA Kit (Single Wash) (AAV9) components.

AAV-X Rapid Titer ELISA Kit (Single Wash) (AAV9) (Store at 4°C)	631487 (96 rxns)
AAV-X Rapid Titer ELISA Components (Cat. No. 631481)*	
Anti-AAV Coated Plate (96 wells)	1 each
AAV HRP-Detection Antibody	12 ml
Washing Buffer (20X)	50 ml
TMB Substrate	12 ml
Stop Solution	12 ml
AAV-X Rapid Titer ELISA Control (AAV9) (Cat. No. 631484)*	
Lyophilized AAV9 Empty Particle Control	2 x 1xE10 VP
Reconstitution Buffer	2 x 1.5 ml
Diluent Buffer	2 x 20 ml

*Not sold separately.

III. Additional Materials Required (Not Provided)

- Latex gloves, safety glasses, and other appropriate protective garments

- Biohazard infectious waste containers
- Micropipettes for delivering volumes of 2 µl, 20 µl, 100 µl, 200 µl, and 1,000 µl
- Filtered pipetting devices for 1 ml, 5 ml, 10 ml, and 25 ml pipettes
- 1.5 ml microcentrifuge tubes
- 25 ml reagent reservoirs
- Automatic microtiter plate washer or a vacuum line fitted with a vacuum pump trap to collect liquid
- Microtiter plate reader with 450 nm filter
- Absorbent paper towels
- Standard laboratory equipment

IV. General Considerations

- The AAV Rapid Titer (Single Wash) kits contain reagents which are optimized and balanced for each kit lot. Do not interchange reagents from kits with different lot numbers. Do not interchange vial caps or stoppers either within or between kits.
- Allow foil bags to warm to room temperature (18–25°C) before opening. This avoids condensation on the inner surface of the bag, which may contribute to a deterioration of the microtiter strips intended for future use.
- Reagents should be dispensed with the tip of the micropipettes touching the side of the well at a point about midsection. Follow manufacturer's recommendations for automatic processors.
- Count the strips according to the number of test samples and install the strips. Make sure the strips are tightly snapped into the plate frame. Return excess strips to the foil pouch containing the desiccant pack and reseal.
- Always keep the upper surface of the microtiter strips free from excess fluid droplets. Reagents and buffer overspill should be blotted dry on completion of the manipulation.
- Do not allow the wells to completely dry during an assay.

V. Protocol: AAV-X Rapid Titer ELISA Kit (Single Wash) (AAV2/8/9) Procedures

NOTES:

– Please read the entire protocol before starting.
– This protocol is applicable to all three AAV serotypes (AAV2, AAV8, and AAV9) available in the AAV Rapid Titer ELISA Kits (Single Wash) (Cat. Nos. 631485, 631486, and 631487). The term "AAV" is used generically in the protocol below to refer to each of the respective serotypes.
– Before use, allow all reagents to reach room temperature (18–25°C).
– If multiple samples are processed at once, we recommend preparing your sample using a *non-binding* 96-well microplate (e.g., Greiner Bio-One, Cat. No. 650901). This modification allows efficient processing using a multichannel pipette while minimizing sample variability during the preparation step.
The reagent amounts prepared in non-binding microcentrifuge tubes in Section V.A.2 and V.A.3, Steps 2–7 are sufficient for performing duplicate assays. When using a non-binding 96-well microplate, reduce all listed solution volumes in Section V.A.2 and V.A.3, Steps 2–7 by half, or as needed.

A. Protocol: Prepare Reagents

1. Protocol: 1X Washing Buffer Preparation

NOTE: If precipitate or crystals are present in Washing Buffer (20X), warm to 37°C and mix until completely dissolved prior to dilution.

Prepare 1X Washing Buffer by diluting 1 part Washing Buffer (20X) with 19 parts Milli-Q water. For example, dilute 10 ml of Washing Buffer (20X) with 190 ml of Milli-Q water to produce 200 ml of 1X Washing Buffer.

2. Protocol: AAV Sample Preparation

To obtain accurate AAV titers, we recommend the following dilution procedure for virus samples.

- Initial Dilution
  - Low Titer: Use the virus sample either as is or diluted 1:10 with Diluent Buffer for a total volume of 500 µl.
  - High Titer: Dilute the virus sample 1:100 or 1:1,000 with Diluent Buffer for a total volume of 500 µl.
- Serial Dilutions
  - Following the initial dilution, perform several 1:1 dilutions using 250 µl of Diluent Buffer per dilution. Once you have run your diluted samples together with the standard curve prepared in Step 3 (below), check that at least one sample dilution falls within the concentration range of the standard curve.

Mix dilutions thoroughly before assaying or diluting them further, assay each sample in duplicate, and be sure to multiply each result by its dilution factor to determine the correct AAV value in the original sample.

3. Protocol: AAV Standard Curve Preparation

To accurately determine AAV titers, you will need to generate a standard curve. Follow the instructions below to prepare a dilution series, which will be used to generate a standard curve in Section V.B, Step 11.

1. Prepare an AAV stock solution (1 x 10^{10} VP/ml) by adding 1,000 µl of Reconstitution Buffer to the Lyophilized AAV Empty Particle Control. Vortex for 5 sec.
2. Prepare an AAV standard solution (4 x 10^{9} VP/ml) by mixing 400 µl of the AAV stock solution (prepared in Step 1) with 600 µl of Diluent Buffer. Vortex for 5 sec. See Figure 2 below for a visual aid of Steps 1–2.

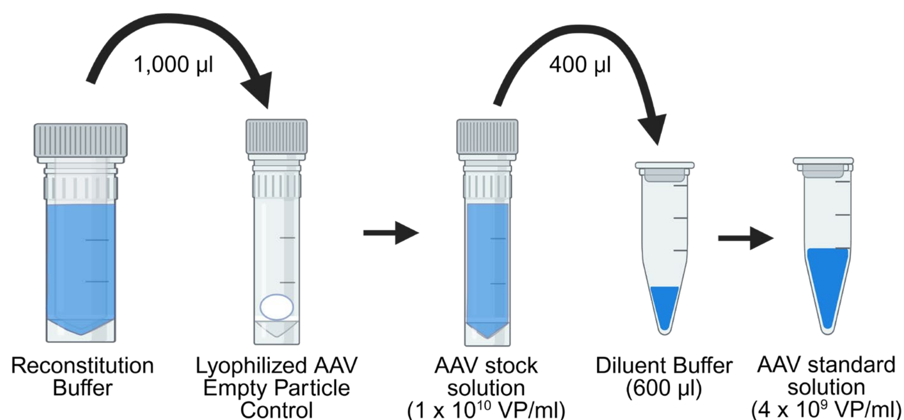

Figure 2. Preparation of the AAV stock solution and AAV standard solution.

3. Label seven 1.5 ml microcentrifuge tubes to create the AAV standard curve with 2 x 10^9, 1 x 10^9, 5 x 10^8, 2.5 x 10^8, 1.25 x 10^8, 6.25 x 10^7 VP/ml, respectively, and the negative control (NTC; 0 VP/ml).
4. Dispense 250 µl of Diluent Buffer into each of the seven 1.5 ml microcentrifuge tubes, then set aside the negative control tube.
5. Begin preparing the AAV standard curve by pipetting 250 µl of the AAV standard solution (4 x 10^9 VP/ml, prepared in Step 2) into the 2 x 10^9 VP/ml tube. Vortex for 5 sec.
6. Add 250 µl of the solution from the 2 x 10^9 VP/ml tube into the 1 x 10^9 VP/ml tube. Vortex for 5 sec.
7. Repeat Step 6 four more times to finish preparing the AAV standard curve. See Figure 3 (below) for a visual aid of Steps 4–7.

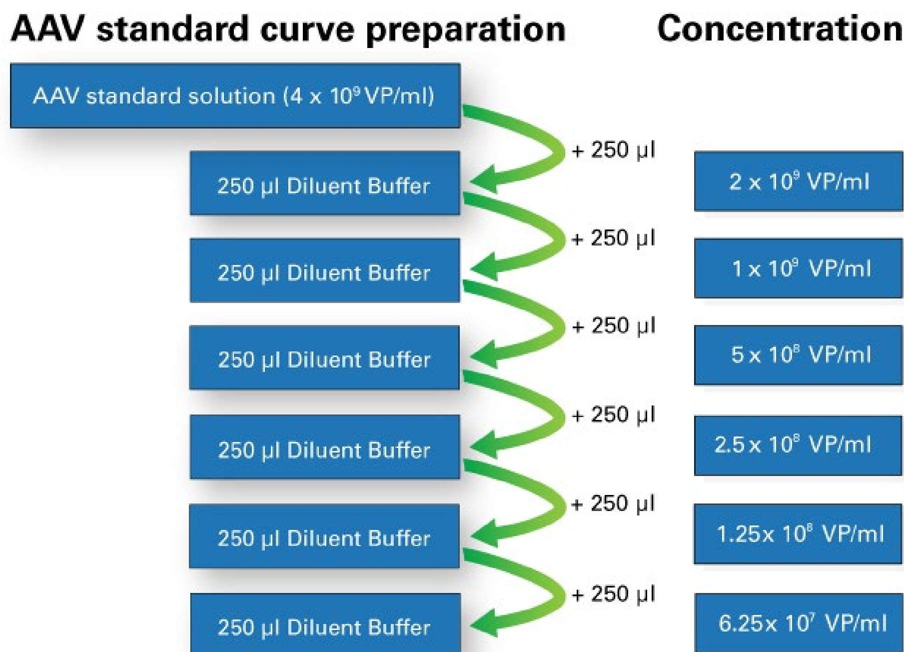

Figure 3. Preparing the dilution series for the AAV standard curve. 250 µl of the AAV standard solution is added to the 2 x 10^9 VP/ml tube containing 250 µl Diluent Buffer. Mix the tube and transfer 250 µl into the next tube (1 x 10^9 VP/ml) containing 250 µl Diluent Buffer. Follow the same dilution process with the remaining tubes.

B. Protocol: Perform ELISA

1. Transfer 100 µl from each of the AAV standard curve and sample dilutions into wells of the Anti-AAV Coated Plate (ELISA plate). Begin measuring the incubation time for Step 2 immediately after adding the final sample.
2. Incubate the ELISA plate at room temperature (18–25°C) for 30 min ± 2 min.
3. Add 100 µl of AAV HRP-Detection Antibody to each well, then gently agitate the plate to mix. Begin measuring incubation time for Step 4 immediately after adding AAV HRP-Detection Antibody to the final well.
4. Incubate the ELISA plate at room temperature (18–25°C) for 30 min ± 2 min.
5. Aspirate the contents of the wells and wash the ELISA plate via either manual or automatic plate washing.
   a. **Manual Plate Washing**

      For manual plate washing, perform the following steps for the rinse cycle:

      i. Use a vacuum line fitted with a vacuum pump trap to aspirate liquid from the sides of all wells.
      ii. With a multichannel pipette or a laboratory squeeze bottle, fill wells with ~300 µl of 1X Washing Buffer (to the brim).
      iii. Aspirate the wells with the vacuum line.
      iv. Repeat steps ii. and iii. five more times (Total: 6 rinse/wash cycles)
      v. Flip the plate well-side down and tap firmly on a paper towel to remove any excess liquid.

   b. **Automatic Plate Washing**

      For automatic plate washing, perform the following steps:

      i. Run a rinse cycle consisting of six consecutive washes.
      ii. After the final rinse cycle, flip the plate well-side down and tap firmly on a paper towel.
      iii. Check for any residual 1X Washing Buffer in the wells and blot the upper surface with a dry paper towel.

   Automatic plate washers should meet the following criteria:

   - All wells must be completely aspirated.
   - All wells are filled with ~300 µl 1X Washing Buffer during each rinse cycle.
   - The apparatus must be regularly cleaned following the manufacturer's instructions to prevent contamination.
6. After washing, immediately dispense 100 µl of TMB Substrate into each well.
7. Incubate the plate in the dark at room temperature (18–25°C) for 15 min ± 2 min.
8. Add 100 µl of Stop Solution to each well to stop the reaction and mix gently. The blue solution should change to a yellow color, in proportion to the different amounts of AAV in the respective sample wells.
9. Ensure that the undersides of the wells are dry and that there are no air bubbles in the wells.
10. Immediately read the absorbance values at 450 nm using a microtiter plate reader.

11. Create the standard curve based on the absorbance values from the AAV of the control dilution series, then use the standard curve to determine the AAV concentration of your samples. For more information on this step, refer to Section VI, "Interpretation of ELISA Reading".

VI. Interpretation of ELISA Reading

A. Collecting Data

1. Run ELISA for samples and AAV standard curve with appropriate replicates, as described in Section V.B.
2. Calculate the mean absorbance for each standard and each sample.
3. (Optional) Subtract the blank from all readings.

B. Plotting the Standard Curve

1. Plot the standard curve using the following axes:
   - X-axis: AAV concentration (VP/ml) for each of the dilutions for the AAV standard curve
   - Y-axis: mean absorbance for each of the dilutions for the AAV standard curve
2. Use a linear regression program to fit a straight line to the standard curve data points based on the following model:

$$y = mx + b$$

   where m is the slope and b is the y-axis intercept.
3. Quantify sample AAV concentrations.

NOTE: Before quantifying, ensure each sample's mean absorbance falls within the standard curve range.

   - Calculate the AAV concentration in each diluted sample using the inverse of the linear model:

$$x = \frac{y - b}{m}$$

     where y is the sample's mean absorbance and x is the sample AAV concentration (VP/ml).
   - Apply dilution factors as necessary to obtain the final sample AAV concentration (VP/ml).

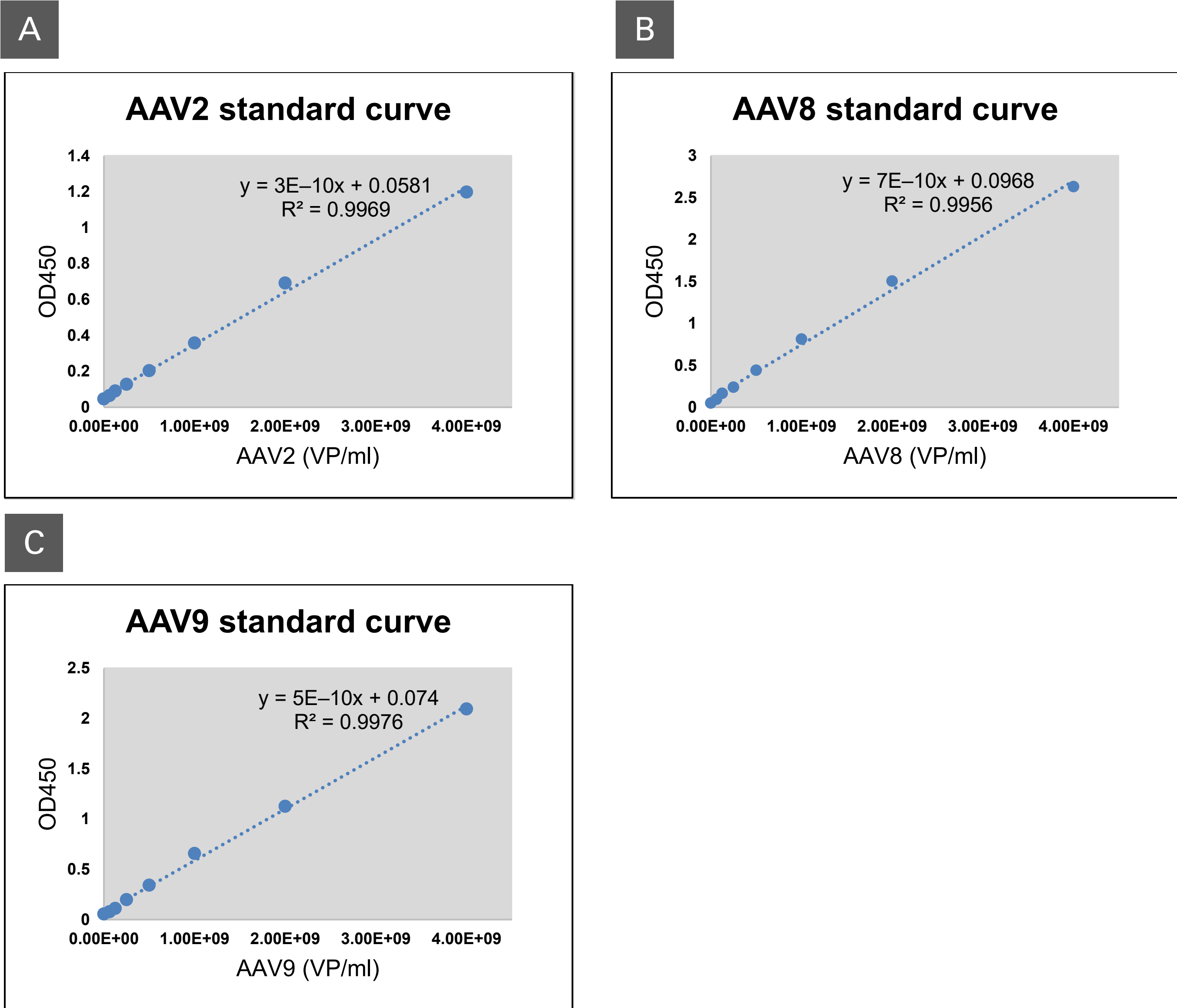

Figure 4. Example standard curves for AAV2, AAV8, and AAV9. Standard curve dilutions were prepared by using the lyophilized AAV2 (**Panel A**), AAV8 (**Panel B**), and AAV9 (**Panel C**) Empty Particle Controls, assayed with corresponding AAV Rapid Titer ELISA Kits (Single Wash). For the standard curves, R^2 values (dashed lines) are based on linear regression analysis.

NOTE: The standard curve provided is for demonstration purposes only. A new standard curve must be prepared each time an assay is performed.

Table 4. Example limits of detection (LOD) and limits of quantification (LOQ).

	AAV2	AAV8	AAV9
LOD (VP/ml)	2.58E+07	7.36E+06	8.59E+06
LOQ (VP/ml)	7.81E+07	2.23E+07	2.60E+07

Appendix A. Troubleshooting Guide

Table 5. Troubleshooting guide.

Problem	Possible Explanation	Solution
Reagents become visibly cloudy or contain a precipitate.	The Washing Buffer (20X) can develop a crystalline precipitate during storage at 2–8°C.	Precipitate will dissolve upon warming the Washing Buffer (20X) to 37°C.
	For other reagents, this may be caused by a kit with previously opened reagents or past its expiration date.	Use a new kit with fresh reagents. If this is a newly opened kit not past the expiration date, contact Technical Support.
The TMB Substrate solution becomes dark blue in color.	This is likely caused by chemical contamination of the substrate solution.	Use a new kit with fresh reagents. If this is a newly opened kit not past the expiration date, contact Technical Support.
The assay cannot be used to quantitate samples having AAV values greater than the highest value on the AAV standard curve.	The samples are not diluted sufficiently	Perform serial dilution of the sample until the dilution falls into the AAV standard curve.

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This document has been reviewed and approved by the Quality Department.