

For Research Use

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# TAKARA

## AAVpro<sup>®</sup> Titration Kit (for Real Time PCR) Ver.2

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Product Manual

We have begun the process of changing the names for Takara Bio's intercalator-based real-time PCR (qPCR) products to the "TB Green series". These products can be used the same way as before, as only the names are changing. Catalog number and product performance are unaffected by this transition.

The long-term storage temperature of TB Green<sup>®</sup> *Premix Ex Taq*<sup>™</sup> II (Tli RNaseH Plus) in this product has been changed to -20°C since Lot. #AH97918A. See section III. Storage.

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**Safety & Handling of Adeno-Associated Virus Vectors**

The protocols in this User Manual require the handling of adeno-associated virus vectors. It is imperative to fully understand the potential hazards of and necessary precautions for laboratory use of these vectors.

Viruses produced with AAV-based vectors could, depending on your gene insert, be potentially hazardous. Similar vectors have been approved for human gene therapy trials, attesting to their potential ability to express genes *in vivo*. For these reasons, due caution must be exercised in the production and handling of any recombinant viruses.

Follow all applicable guidelines for research involving recombinant DNA. Take appropriate safety measures when producing or handling recombinant adeno-associated viruses, including working in a biological safety cabinet and wearing protective laboratory coats, face protection, and gloves.

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**Available AAVpro Products**

|   |              |
|---|--------------|
| AAVpro® Helper Free System (AAV2)                   | Cat. #6230   |
| AAVpro® Purification Kit (AAV2)                     | Cat. #6232   |
| AAVpro® Purification Kit (All Serotypes)            | Cat. #6666   |
| AAVpro® Titration Kit (for Real Time PCR) Ver.2     | Cat. #6233   |
| AAVpro® Extraction Solution                         | Cat. #6235   |
| pAAV-ZsGreen1 Vector                                | Cat. #6231   |
| AAVpro® Helper Free System (AAV2-CRE Recombinase)   | Cat. #6652   |
| AAVpro® Helper Free System (AAV2-LacZ)              | Cat. #6655   |
| AAVpro® Tet-One™ Inducible Expression System (AAV2) | Cat. #634310 |

## I. Description

The AAVpro Titration Kit (for Real Time PCR) is a kit for determining the titer of adeno-associated virus (AAV) using real-time PCR. This kit contains all of the reagents necessary for extraction of AAV particles from AAV vector-producing cells and real-time PCR assay of virus genome. This kit allows more precise and rapid quantification than conventional DNA blotting or ELISA methods. Quantification is based on amplification of the ITRs (inverted terminal repeats) of AAV, and therefore titer measurement is possible for various serotypes of AAV. The stability of Positive Control has been improved for Ver.2.

### AAV Vectors

AAV is a non-enveloped virus that belongs to the *Parvovirus* family of the *Dependovirus* genus. The AAV genome is a linear, single-strand DNA molecule of approximately 4.7 kb that has terminal hairpin structures called inverted terminal repeats (ITRs) at both ends.

Recombinant AAV particles exploit the properties of AAV for transduction of genes to cells and organisms. AAV vectors are used as research tools and also as vectors for gene therapy.

The AAVpro Helper Free System (AAV2) (Cat. #6230) can be used to prepare recombinant AAV2 particles by transfecting HEK293 or other cells with plasmids that express the necessary components required for AAV production.

### Measurable AAV Serotypes

The serotype of AAV is determined by the type of Cap gene on the pRC plasmid used to prepare viral particles. The viral genome encapsulated within AAV particles typically contain the ITRs of AAV2, regardless of serotype. This kit uses the ITR sequence of AAV2 as a target to quantify the amount of viral genome. Therefore, it can be used regardless of serotype as long as the ITR domain of the vector genome is of AAV2 origin. Prior to using this kit, confirm that the ITR domain of the AAV vector being used is derived from AAV2.

### Simplified AAV Genome Extraction

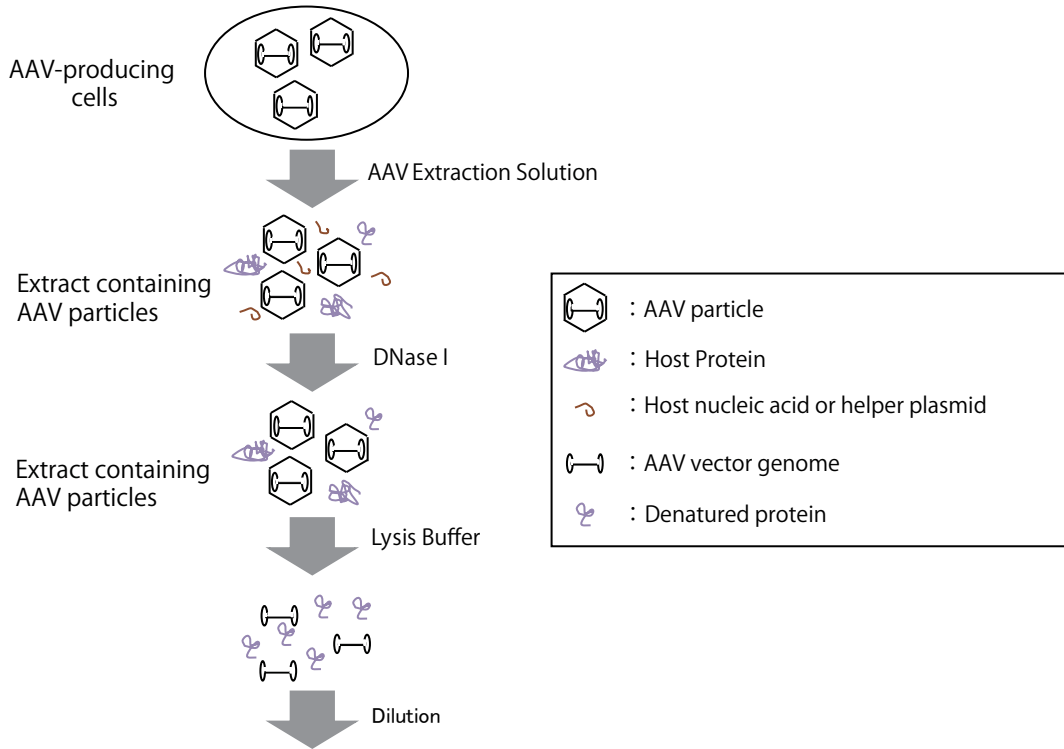
#### (A) AAV Extraction using AAV Extraction Solution

Extraction of AAV particles from AAV-producing cells is conventionally performed using freeze-thaw or sonication methods; however, these methods are time consuming and require special equipment. This kit includes AAV Extraction Solutions\* that allow simple and efficient AAV particle extraction while minimizing protein and nucleic acid contamination.

\* Also sold separately (AAVpro Extraction Solution, Cat. #6235).

#### (B) Extraction of AAV Genome from Extracts Containing Viral Particles

Extracts containing AAV particles also contain proteins and nucleic acids from the producer cells. This kit includes DNase I for removal of free nucleic acids and Lysis Buffer for heat-denaturation of proteins in extracts containing AAV particles.



Vector Genome Assay using Real-Time PCR

Figure 1. Overview of the AAV particle and vector genome extraction.

Real-Time PCR

Real-time PCR can be used for rapid and sensitive detection of viral genomic DNA. In this method, PCR amplification is monitored in real time using a fluorescent DNA intercalator. The reagent in this kit, TB Green *Premix Ex Taq II*, includes a hot start PCR enzyme and TB Green for fluorescent detection.

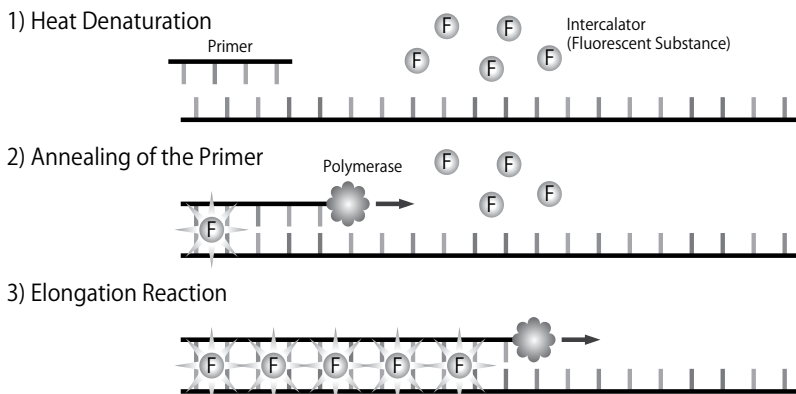


Figure 2. Fluorescent intercalator detection method.

This method involves the addition of an intercalating reagent that emits fluorescence when bound to double-strand DNA in the reaction mixture. This enables the detection of amplified DNA by monitoring fluorescence.

## II. Components

|  |                 |
|--|-----------------|
| 1. Extraction of the AAV Vector                                  |                 |
| AAV Extraction Solution A  | 1.5 ml x 2      |
| AAV Extraction Solution B  | 300 $\mu$ l     |
| 2. Simplified AAV Genome Extraction (for 100 samples)            |                 |
| DNase I  | 100 $\mu$ l     |
| 10X DNase I Buffer   | 200 $\mu$ l     |
| Lysis Buffer   | 1 ml x 2        |
| 3. Real Time PCR (For 100 reactions; 25 $\mu$ l volume)          |                 |
| TB Green <i>Premix Ex Taq</i> II (Tli RNaseH Plus)* <sup>1</sup> | 625 $\mu$ l x 2 |
| AAV Forward Titer Primer   | 20 $\mu$ l      |
| AAV Reverse Titer Primer   | 20 $\mu$ l      |
| dH <sub>2</sub> O* <sup>2</sup>                                  | 1 ml x 3        |
| ROX Reference Dye* <sup>3</sup>                                  | 50 $\mu$ l      |
| ROX Reference Dye II* <sup>3</sup>                               | 50 $\mu$ l      |
| Positive Control   | 50 $\mu$ l      |
| EASY Dilution (for Real Time PCR)                                | 1 ml x 5        |

\* 1 The name of intercalator-based qPCR reagent has been changed.

\* 2 dH<sub>2</sub>O is also used in the DNase I reaction.

\* 3 Use when reactions will be performed on an Applied Biosystems real-time PCR device or another instrument that uses between-well correction of the fluorescent signal.

◆ Add ROX Reference Dye

• StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)

◆ Add ROX Reference Dye II

• Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific)

◆ No ROX Reference Dye

• Thermal Cycler Dice™ Real Time System series (Cat. #TP900/TP960, etc.)\*<sup>4</sup>

\* 4 Not available in all geographic locations. Check for availability in your area.

## III. Storage

- TB Green *Premix Ex Taq* II  
Store at 4°C for up to 6 months. Protect from light and avoid contamination.  
\* Keep at -20°C for long term storage. Once thawed, store at 4°C and use within 6 months.
- AAV Extraction Solution A and B and Lysis Buffer  
-20°C; store at room temperature after thawing.
- Other components  
-20°C. Use the components other than TB Green *Premix Ex Taq* II within 2 years.

#### IV. Materials Required but Not Provided

- General equipment for cell culture
- Transfection reagent (e.g., CalPhos™ Mammalian Transfection Kit, Cat. #631312; Xfect™ Transfection Reagent, Cat. #631317)
- 0.5 M EDTA (pH 8.0) (EDTA Buffer Powder, pH 8.0 (Cat. #T9191))
- Heat block (set to 37°C, 70°C, 95°C), or a thermal cycler
- Real-time PCR instrument and tubes
  - Thermal Cycler Dice Real Time System // (Cat.#TP900/TP960)\*
  - Thermal Cycler Dice Real Time System *Lite* (Cat.#TP700/TP760)\*
  - Applied Biosystems 7500 Fast Real Time PCR System (Thermo Fisher Scientific)
  - StepOnePlus Real Time PCR System (Thermo Fisher Scientific), etc.

\* Not available in all geographic locations. Check for availability in your area.

#### V. Precautions for Use

The following are precautions regarding the use of this kit. **Read before use.**

1. This kit includes reaction reagents for the extraction of AAV particles from AAV-producing cells, the extraction of the vector genome from AAV particles, and real-time PCR.
2. Store the primer in the kit at -20°C without mixing or dilution, and prepare the primer mix immediately before use.
3. For the PCR reaction mixture, prepare a master mix that contains dH<sub>2</sub>O, buffer, and enzyme. Preparing a master mix will decrease the loss due to pipetting and increase accuracy and reproducibility.
4. Briefly centrifuge TB Green *Premix Ex Taq II* before use to bring all of the reagent to the bottom of the tube. Pipette slowly and carefully, as it is highly viscous.
5. When dispensing reagents, be sure to use a new disposable tip, and avoid between-sample contamination.
6. When preparing reaction solutions and samples and adding samples to the reaction solution, physically separate the areas and avoid opening and closing tubes that contain amplification products. Because the amplification reaction and detection take place in real time, there is no need for electrophoresis on the amplification products after the reaction is complete.

## VI. Protocol

Note: The following procedures include the process of extracting AAV particles from AAV-producing cells. To measure the titer of AAV that has already been isolated, begin the protocol at step VI-2.

### VI-1. Extraction of AAV Particles

The following protocol is for extraction from one 10-cm dish containing AAV vector-producing cells. Refer to Table 1 for the amount of solution that should be used for different types of culture vessels.

1. Add 125  $\mu$ l of 0.5 M EDTA (pH 8.0) (1/80 volume of the culture media) to a 10-cm dish containing AAV vector-producing cells (10-ml culture volume). Refer to Table 1 for the amount of EDTA for different types of culture vessels. Allow to stand at room temperature for 10 min.
2. Transfer the detached cells to a sterile 15-ml centrifuge tube.
3. Centrifuge at 1,750g at 4°C for 10 min. Completely remove the supernatant and collect the cell pellet.

**Note 1:** Confirm that the supernatant has been completely removed before proceeding; particle extraction may be affected by residual supernatant.

4. Loosen the cell pellet by tapping or vortexing the tube.

**Note 2:** If the cell pellet has not been loosened sufficiently, the efficiency of extraction may decrease. Confirm that there are no clumps of cells before proceeding.

5. Add 0.5 ml of AAV Extraction Solution A.
6. Suspend the cell pellet by vortexing for 15 sec.
7. Allow to stand at room temperature for 5 min. Vortex for 15 sec.
8. Centrifuge at 2,000 - 14,000g at 4°C for 10 min to pellet cell debris.

**Note 3:** If the titer of the recovered AAV vector is low, the efficiency may be increased by repeating steps 6 - 8.

9. Collect the supernatant in a new sterile centrifuge tube. Add 50  $\mu$ l of AAV Extraction Solution B and mix by pipetting.

**Note 4:** The mixture can be stored at -80°C. Thaw quickly in a 37°C water bath before use.

**Note 5:** The supernatant may change to a pink color after AAV Extraction Solution B is added.

Table 1. Amount of solutions required for various culture vessels.

|            | Volume of Culture | 0.5 M EDTA (pH 8.0) | AAV Extraction Solution A | AAV Extraction Solution B |
|------------|-------------------|---------------------|---------------------------|---------------------------|
| 6-cm dish  | 4 ml              | 50 $\mu$ l          | 200 $\mu$ l               | 20 $\mu$ l                |
| 10-cm dish | 10 ml             | 125 $\mu$ l         | 500 $\mu$ l               | 50 $\mu$ l                |
| 15-cm dish | 26 ml             | 325 $\mu$ l         | 1,300 $\mu$ l             | 130 $\mu$ l               |
| T25 flask  | 4 ml              | 50 $\mu$ l          | 250 $\mu$ l               | 25 $\mu$ l                |
| T75 flask  | 13 ml             | 162.5 $\mu$ l       | 650 $\mu$ l               | 65 $\mu$ l                |
| T225 flask | 40 ml             | 500 $\mu$ l         | 2,000 $\mu$ l             | 200 $\mu$ l               |



**VI-2. Extraction of AAV Vector Genome**

1. Treat the AAV particle solution with DNase I and incubate at 37°C for at least 15 min to digest free genomic DNA and plasmid DNA derived from host cells.

|                       |            |
|-----------------------|------------|
| AAV Particle Solution | 2 $\mu$ l  |
| 10X DNase I Buffer    | 2 $\mu$ l  |
| DNase I               | 1 $\mu$ l  |
| dH <sub>2</sub> O     | 15 $\mu$ l |
| Total                 | 20 $\mu$ l |

2. Inactivate DNase I by heat treatment at 95°C for 10 min.
3. Add an equal amount of Lysis Buffer (20  $\mu$ l).
4. Incubate at 70°C for 10 min.
5. Dilute the AAV vector genome solution at least 50-fold using EASY Dilution (for Real Time PCR) and use directly as the template for real-time PCR.

**VI-3. Real-Time PCR**

Use 5  $\mu$ l of the AAV vector genome solution prepared in VI-2. as the template for real-time PCR. At the same time, use the Positive Control to prepare the standard curve.

1. Sample Preparation for Standard Curve

Dilute the Positive Control using EASY Dilution to obtain the samples for standard curve preparation. (Use 5  $\mu$ l of each solution as a template for real-time PCR.)

- (1)  $2 \times 10^7$  copies/ $\mu$ l (Positive Control solution)
- (2)  $2 \times 10^6$  copies/ $\mu$ l (5  $\mu$ l of Positive Control solution + 45  $\mu$ l of EASY Dilution)
- (3)  $2 \times 10^5$  copies/ $\mu$ l (5  $\mu$ l of (2) + 45  $\mu$ l of EASY Dilution)
- (4)  $2 \times 10^4$  copies/ $\mu$ l (5  $\mu$ l of (3) + 45  $\mu$ l of EASY Dilution)
- (5)  $2 \times 10^3$  copies/ $\mu$ l (5  $\mu$ l of (4) + 45  $\mu$ l of EASY Dilution)
- (6)  $2 \times 10^2$  copies/ $\mu$ l (5  $\mu$ l of (5) + 45  $\mu$ l of EASY Dilution)

2. Preparation of 50X Primer Mix

Prepare the 50X primer mix as indicated below. Prepare the necessary amount of 50X primer mix immediately prior to use. Do not store.

|                          |            |
|--------------------------|------------|
| AAV Forward Titer Primer | 5 $\mu$ l  |
| AAV Reverse Titer Primer | 5 $\mu$ l  |
| TE or dH <sub>2</sub> O  | 15 $\mu$ l |
| Total                    | 25 $\mu$ l |

## 3. Preparation of the Reaction

Prepare the reaction mixtures shown below on ice.

Prepare a master mix containing all of the components except for the template. Prepare sufficient master mix for the required number of tubes plus a few extra, and dispense 20  $\mu$ l into each reaction tube and close the caps lightly.

(For Thermal Cycler Dice Real Time System)

[ 1 reaction]

|   |               |
|---|---------------|
| TB Green <i>Premix Ex Taq</i> II (2X conc.) | 12.5 $\mu$ l  |
| 50X Primer mix                              | 0.5 $\mu$ l   |
| dH <sub>2</sub> O                           | 7.0 $\mu$ l   |
| Template                                    | (5.0 $\mu$ l) |
| Total                                       | 25.0 $\mu$ l  |

(For Applied Biosystems Real Time PCR Equipment)

[ 1 reaction]

|   |               |
|---|---------------|
| TB Green <i>Premix Ex Taq</i> II (2X conc.) | 12.5 $\mu$ l  |
| 50X Primer mix                              | 0.5 $\mu$ l   |
| ROX Reference Dye or ROX Reference Dye II*  | 0.5 $\mu$ l   |
| dH <sub>2</sub> O                           | 6.5 $\mu$ l   |
| Template                                    | (5.0 $\mu$ l) |
| Total                                       | 25.0 $\mu$ l  |

\* For StepOnePlus, use ROX Reference Dye, and for Applied Biosystems 7500 Fast Real-Time PCR System, use ROX Reference Dye II.

## 4. Addition of the Template

Add 5  $\mu$ l of dH<sub>2</sub>O to one tube as the negative control and close the cap of the reaction tube tightly.

Add 5  $\mu$ l of the template (the sample DNA solution and standards) to the reaction solutions and close the caps tightly. Wear gloves to prevent getting the tubes dirty when closing the caps, as fluorescence will be measured.

Centrifuge briefly and place in the real-time PCR amplification device.

## 5. Start the Real-Time PCR Reaction

Use the following PCR conditions.

\* Please refer to the instruction manual for the real-time PCR amplification device for specific operation methods.

Initial Denaturation  
95°C 2 min

2 Step PCR

35 Cycles

95°C 5 sec

60°C 30 sec (Detection of Fluorescence: FAM)

Melt Curve Analysis

## VII. Quantification

The Positive Control in this kit is a plasmid DNA that includes the ITR, adjusted to  $2 \times 10^7$  copies/ $\mu$ l based on the value at OD<sub>260</sub>. When serially diluted, it can be used to produce a standard curve for absolute quantification. Because the control contains the same ITR copy number as the AAV vector genome, it is possible to convert copy number to vector genome number (vg/ml and vg/cell). This kit is improved at stability of Positive Control.

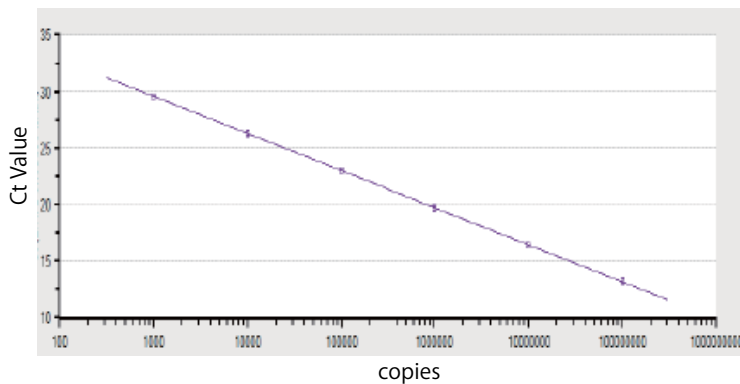


Figure 3. Standard Curve generated with the Positive Control.

## VIII. Experimental Examples

### Experiment 1: Vector Genome Quantification using ITR and Other Regions

Real-time PCR amplification of either the ITR domain (target in this kit) or the CMV promoter region of the AAV vector genome was performed to quantify vector genomes.

(Methods)

HEK293 cells producing AAV1, AAV2, and AAV6 vectors were prepared. AAV vectors were extracted from these cells using AAV Extraction Solution and quantitative analysis was performed using real-time PCR. The ITR and the CMV promoter regions of the AAV vector genome were amplified and the titer was calculated (Figure 4).

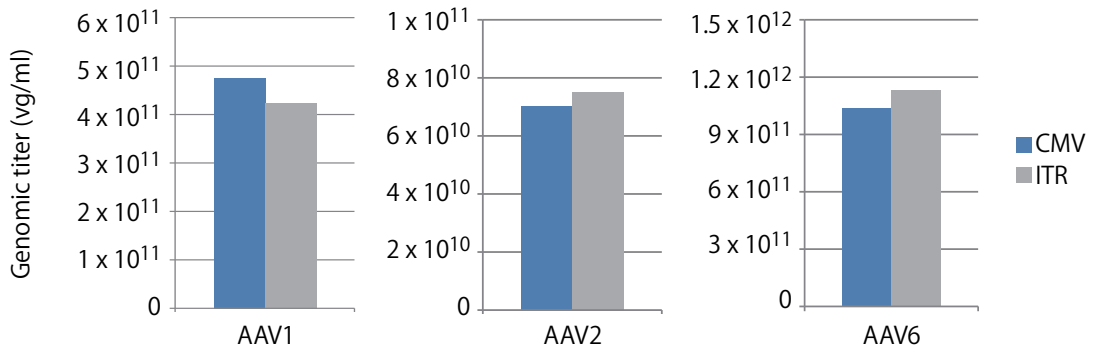


Figure 4. Titer measurement of AAV by real-time PCR using ITR and CMV promoter targets.

(Results)

The titer was roughly identical for both the ITR and CMV promoter targets.

**Experiment 2: Comparison of AAV Extraction Solution with the Freeze-Thaw Method**

AAV vector was extracted from AAV-producing cells using either the AAV Extraction Solution in this kit or the conventional freeze-thaw method.

(Methods)

HEK293 cells producing AAV1, AAV2, and AAV6 vectors were prepared, and AAV vector was extracted from the cells using the AAV Extraction Solution or the freeze-thaw method. Quantitative analysis of virus titer was performed by real-time PCR using this kit (Figure 5).

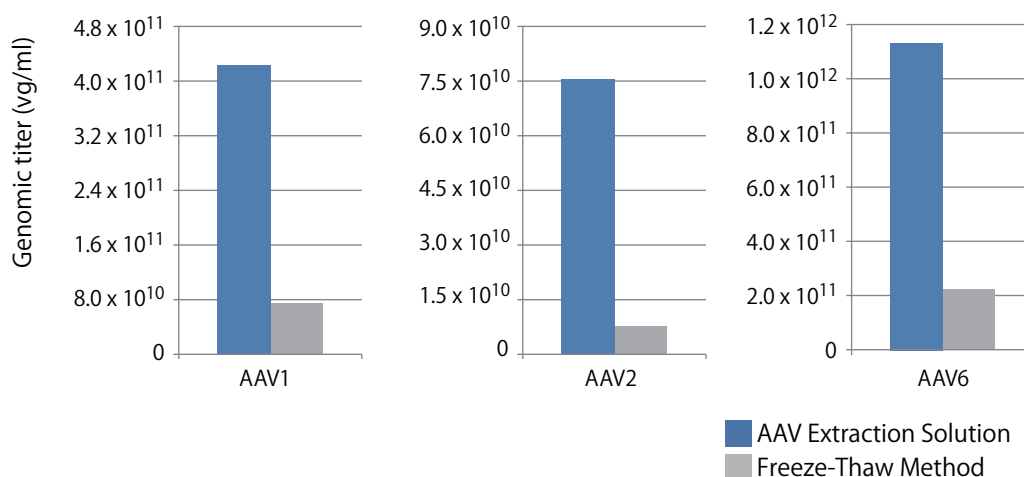


Figure 5. Titer of AAV particles prepared with AAV Extraction Solution or the freeze-thaw method.

(Results)

The use of AAV Extraction Solution for AAV preparation provided higher extraction efficiency than the freeze-thaw method, generating >5X higher titers in this experiment.

**IX. Related Products**

AAVpro® Helper Free System (Cat. #6230)

pAAV-ZsGreen1 Vector (Cat. #6231)

AAVpro® Purification Kit (AAV2) (Cat. #6232)

AAVpro® Extraction Solutions (Cat. #6235)

AAVpro® Helper Free System (AAV2-CRE Recombinase) (Cat. #6652)

AAVpro® Helper Free System (AAV2-LacZ) (Cat. #6655)

AAVpro® Tet-One™ Inducible Expression System (AAV2) (Cat. #634310)

Thermal Cycler Dice™ Real Time System // (Cat. #TP900/TP960)\*

Thermal Cycler Dice™ Real Time System *Lite* (Cat. #TP700/TP760)\*

EASY Dilution (for Real Time PCR) (Cat. #9160)

\* Not available in all geographic locations. Check for availability in your area.

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