Cat. # 6676 - 6678

For Research Use

TaKaRa

AAVpro® Cell & Sup. Purification Kit Maxi (All Serotypes) **(Cat. #6676)**

AAVpro® Cell & Sup. Extraction/Concentration Pack Maxi (All Serotypes) **(Cat. #6677)**

AAVpro® Purification Pack Maxi (All Serotypes) **(Cat. #6678)**

Product Manual

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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.

I. Introduction

Adeno-Associated Virus (AAV) is a non-enveloped virus that belongs to the Parvovirus family of the Dependovirus genus. There are more than 100 serotypes of AAV, and the host specificity and characteristics of the virus differ among serotypes. AAV is not thought to be pathogenic to humans and only replicates in the presence of a helper virus, such as adenovirus or herpesvirus. The AAV genome is a linear, single-strand DNA molecule of approximately 4.7 kb.

Adeno-associated virus vectors (AAV vectors) 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. In addition, AAV vectors are generally considered safer than adenoviral and retroviral vectors. AAV vectors can be used to transduce genes into both proliferating and non-proliferating cells and can impart long-term expression in non-dividing cells. In addition, AAV has little immunogenicity and is suitable for the transduction of genes into animals (as an *in vivo* transduction tool).

II. Description

The purity and titer of the AAV particles are important factors for obtaining efficient and stable gene transfer in cultured cells and individual animals. Cesium chloride density gradient centrifugation and iodixanol ultracentrifugation are conventional methods used to purify AAV vectors. However, these methods are time consuming, and require specialized equipment and advanced techniques.

The AAVpro Cell & Sup. Purification Kit Maxi (All Serotypes) allows for simple and fast AAV particle purification from both AAV-producing cells and culture supernatant in about 7 hours.

- Simultaneous high-purity, high-yield AAV particle purification from both AAV-producing cells and culture supernatant
- Can be used for any AAV serotype
- Unique AAV particle extraction method eliminates time-consuming steps such as freeze-and-thaw or sonication
- No complicated techniques such as ultracentrifugation
- Contains all buffers required to purify AAV particles

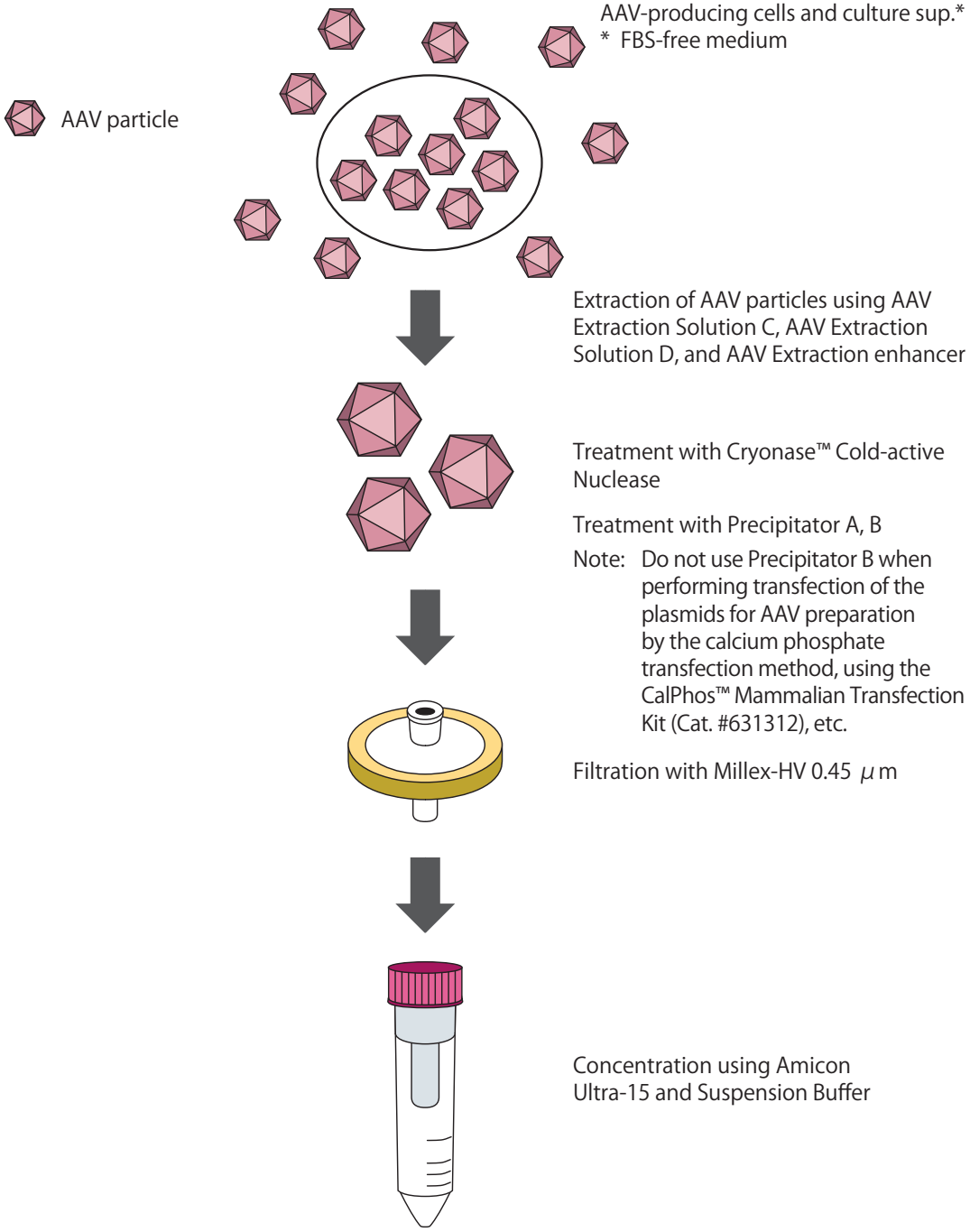


Figure 1. Purification of AAV particles using AAVpro Cell & Sup. Purification Kit Maxi (All Serotypes)

III. Components

- AAVpro Cell & Sup. Purification Kit Maxi (All Serotypes) (Cat. #6676) consists of AAVpro Cell & Sup. Extraction/Concentration Pack Maxi (All Serotypes) (Cat. #6677) and AAVpro Purification Pack Maxi (All Serotypes) (Cat. #6678).
  This kit contains sufficient buffer and columns for four AAV particle purifications, each from five T225 flasks of AAV-producing cells and culture supernatant.
- Cat. #6677 and 6678 are also sold separately.

AAVpro Cell & Sup. Purification Kit Maxi (All Serotypes) (Cat. #6676)

AAVpro Cell & Sup. Extraction/Concentration Pack Maxi (All Serotypes) (Cat. #6677)
AAVpro Purification Pack Maxi (All Serotypes) (Cat. #6678)

AAVpro Cell & Sup. Extraction/Concentration Pack Maxi (All Serotypes) (Cat. #6677)

AAV Extraction Solution C	8 ml
AAV Extraction Solution D	32 ml
AAV Extraction enhancer	80 ml
Concentrating solution*1	115 ml x 2
Dissolving solution	50 ml

AAVpro Purification Pack Maxi (All Serotypes) (Cat. #6678)

Precipitator A*2	5 ml
Precipitator B	2.5 ml
Millex-HV 0.45 μm	4 pcs
Amicon Ultra-15, 100 kDa	4 pcs
Suspension Buffer	60 ml x 2
Cryonase Cold-active Nuclease	500 μl

*1 Precipitate may form when Concentrating solution is stored at 4℃, but this will not affect the quality or performance of this reagent. If precipitate is present, warm to 40℃ and completely dissolve any precipitate before use.

*2 A white precipitate may form in Precipitator A when it is stored at low temperatures, but this will not affect the quality or performance of this reagent. If precipitate is present, warm to 37℃ and completely dissolve any precipitate before use.

IV. Storage

AAVpro Cell & Sup. Extraction/Concentration Pack Maxi (All Serotypes) (Cat. #6677)

Concentrating solution	4℃
Other components	Room temperature

AAVpro Purification Pack Maxi (All Serotypes) (Cat. #6678)

Cryonase Cold-active Nuclease	-20℃
Other components	Room temperature

V. Materials Required but not Provided

- General equipment necessary for cell culture
- Syringes

VI. Protocol

The following protocol describes the purification of AAV particles from producer cells and culture medium in five T225 flasks, each of which contain 40 ml culture medium/flask.

VI-1. Precautions during use

- For transfection of AAV vector plasmids into cells, we recommend using *Trans*IT-VirusGEN Transfection Reagent (Mirus Bio: Code. MIR6700*, MIR6703 - MIR6706*), *Trans*IT-293 Transfection Reagent (Mirus Bio: Code. MIR2700*, MIR2704 - MIR2706*), or CalPhos Mammalian Transfection Kit (Cat. #631312), etc.
  When using other transfection reagents (PEI, etc.), perform a preliminary test to confirm that they are appropriate for use with this kit.
  When using the CalPhos Mammalian Transfection Kit (Cat. #631312) or other calcium phosphate transfection reagents to perform transfection of AAV plasmids, DO NOT perform steps VI-3-3 and VI-3-4 in "VI-3. AAV purification and concentration".

- For extraction and purification of AAV particles in this kit, use AAV-producing cells that have been cultured for 3 to 5 days in culture medium (DMEM, etc.) that does not contain fetal bovine serum (FBS). (Use of culture medium containing serum should be avoided because it results in decreased virus purification efficiency.)

- AAVpro Helper Free System (Cat. #6230, 6650 - 6663*, 6668 - 6671*, 6673) is recommended for preparing AAV particles. When using these kits, AAV-producing cells should be cultured in DMEM that does not contain FBS for 3 to 5 days after transfection of the plasmids for AAV preparation.

- If the AAV particles are excessively concentrated in the concentration step (VI-3-7 onward), they may become insoluble. Take care to ensure that they are not excessively concentrated by centrifugation.

- If you want to check the virus yield before purification, we recommend measuring the titer using part of the AAV particle extract (VI-2-4 Supernatant). AAVpro Titration Kit (for Real-Time PCR) Ver. 2 (Cat. #6233) can be used to measure the titer of the AAV vector.

* Not available in all geographic regions. Please check for availability in your area.

VI-2. Preparation of AAV particle extract

To prepare an AAV particle extract from AAV-producing cells with this kit, AAV Extraction Solution C, AAV Extraction Solution D, and AAV Extraction enhancer are added directly to the culture vessel containing the AAV-producing cells and culture medium. This allows recovery of the AAV particles simultaneously from both the AAV-producing cells and the culture supernatant through concentration of the AAV particle extract using the Concentration solution.

1. To a T225 flask containing AAV-producing cells and culture medium (40 ml), add 1/100 volume (0.4 ml) of AAV Extraction Solution C and 1/25 volume (1.6 ml) of AAV Extraction Solution D, mix gently, and incubate for 10 min at room temperature.
2. Add 1/10 volume (4 ml) of AAV Extraction enhancer and mix gently.
3. Incubate for 30 min at room temperature and then transfer the entire amount, including clumps of cells, to a centrifuge tube.
4. Centrifuge at 2,000*g* for 10 min at 4℃ and recover the supernatant.
   - Note 1: If you do not have a centrifuge with a sufficiently large capacity, divide the supernatant up among a few centrifuge tubes before proceeding to step VI-2-5.
   - Note 2: To check the virus yield, we recommend storing part of the supernatant. AAVpro Titration Kit (for Real-Time PCR) Ver. 2 (Cat. #6233) can be used to measure the titer of the AAV.
5. Add 1/4 volume of Concentrating solution to the supernatant obtained in step VI-2-4, mix well, and incubate at 4℃ for 2 to 3 hr.
   - Note: Although a 2 to 3 hr incubation is recommended, you can also incubate at 4℃ overnight.
6. Centrifuge at 2,000*g* for 10 min at 4℃ and discard the supernatant.
7. Centrifuge again and completely remove the remaining supernatant.
8. Add Dissolving solution (1/20 volume of the supernatant obtained in step VI-2-4) to the pellet from step VI-2-7 and resuspend the pellet by pipetting.
   - Note: Confirm that there are no clumps before proceeding to the next step.
9. Incubate at room temperature for 15 min and then vortex for 15 sec.
10. Centrifuge at 2,000*g* for 10 min at 4℃.
11. Transfer the supernatant to a new sterile centrifuge tube.
    - Note: Make sure that no impurities are transferred along with the supernatant.

VI-3. AAV particle purification and concentration

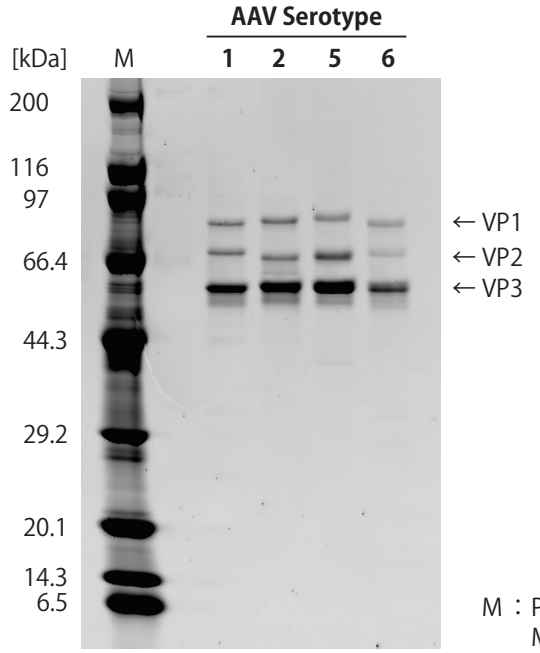
Use swinging bucket rotors for centrifugation in steps VI-3-7, VI-3-8, and VI-3-9.

1. Add 1/100 volume of Cryonase Cold-active Nuclease (final concentration 200 U/ml) to the supernatant from step VI-2-11 and incubate at 37℃ for 1 hr.
2. Add 1/10 volume of Precipitator A and vortex for 10 sec to mix.
   - Note 1: When using the CalPhos Mammalian Transfection Kit (Cat. #631312) or other calcium phosphate transfection reagents to transfect plasmids for AAV production, skip steps VI-3-3 and VI-3-4 and proceed to step VI-3-5.
   - Note 2: Precipitator A may produce a white precipitate at low temperatures, however this does not affect the quality or performance of this reagent. If a precipitate is present, dissolve it completely at 37℃ before use.
   - Note 3: Although a precipitate may form during the reaction, this is not a problem. Proceed to the next step.
3. Incubate at 37℃ for 30 min, and vortex again for 10 sec.
4. Add 1/20 volume of Precipitator B to the solution and promptly vortex for 10 sec to mix.
5. After centrifuging at 5,000 to 9,000*g* for 5 min at 4℃, recover the supernatant.
   - Note: If the recovered supernatant contains a precipitate, centrifuge it again to eliminate as much of the precipitate as possible.
6. Filter the supernatant using a Millex-HV 0.45 μm filter.
7. Transfer the filtered AAV particle solution to an Amicon Ultra-15,100 kDa. Centrifuge at 2,000*g* for 5 min at 15℃ and confirm that the AAV solution volume is ~1.5 ml.
   - Note 1: To avoid excessively concentrating the AAV particle solution, centrifuge for a short period of time (about 1 to 2 min) and then adjust the centrifugation time after checking the volume of the AAV particle solution.
   - Note 2: If the volume of the AAV particle solution is over ~1.5 ml, centrifuge again.
8. After removing the filtrate, add 5 ml of Suspension Buffer to the cup of the Amicon Ultra-15 filter unit and mix by pipetting. Centrifuge at 2,000*g* for 5 min at 15℃, and then confirm that the volume of the AAV solution in the filter is now 1.5 ml or less.
   - Note: If the volume of AAV solution is over ~1.5 ml, centrifuge again.
9. Repeat step VI-3-8 four times (for a total of five times). Concentrate the AAV solution in the filter to the desired volume.
   - Note: If AAV solution is concentrated too much, the AAV particles may become insoluble. While performing centrifugal concentration, stop the centrifuge as appropriate and try pipetting the AAV solution to make sure it has not become insoluble.
10. After removing the filtrate, resuspend the solution inside the cup of the Amicon Ultra-15 filter unit by pipetting or vortexing for 30 sec and transfer the AAV solution to a new tube.
    - Note: To avoid repeatedly freezing and thawing the AAV particle solution, we recommend that you aliquot and store the solution at -80℃.

VII. Experimental Examples

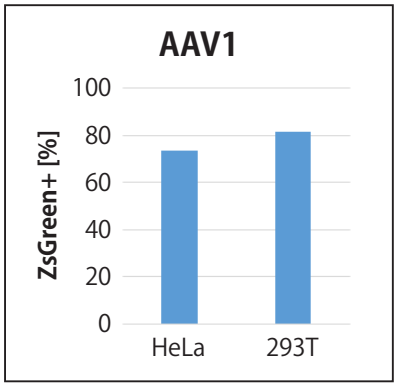
VII-1. Data 1: Purity of AAV particles after purification

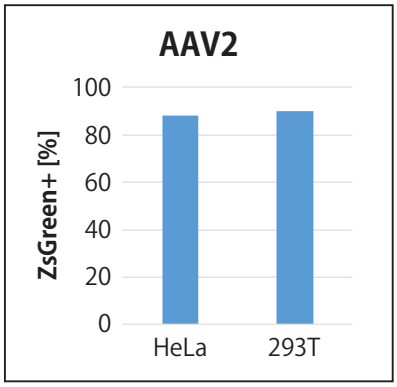
AAV (serotype 1, 2, 5 and 6) particles carrying the florescence protein ZsGreen were extracted from producer cells with this kit. The titers of the purified AAV particles were measured using the AAVpro Titration Kit (for Real Time PCR) Ver.2 (Cat. #6233), and SDS-PAGE was performed using 1 x 10^9 vector genome (vg) per lane. The AAV capsid proteins (VP1, VP2, and VP3) were confirmed to be the major bands present.

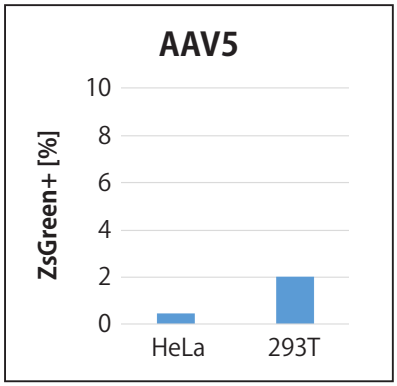


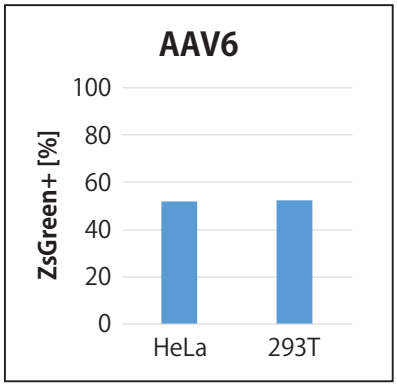
VII-2. Data 2: Infectivity of purified AAV particles

Using the AAV particles of each serotype obtained in Data 1, the infectious titers were evaluated. Cells were infected with the purified AAV particles at 50,000 vg/cell, and flow cytometry analysis was performed after 3 days. It was confirmed that the AAV particles purified with this kit retained their infectivity.









VIII. Related Products

AAVpro® Helper Free System (AAV1) (Cat. #6673)
AAVpro® Helper Free System (AAV2) (Cat. #6230)
AAVpro® Helper Free System (AAV5) (Cat. #6650)
AAVpro® Helper Free System (AAV6) (Cat. #6651)
AAVpro® Helper Free System (AAV1-CRE Recombinase) (Cat. #6668)
AAVpro® Helper Free System (AAV2-CRE Recombinase) (Cat. #6652)
AAVpro® Helper Free System (AAV5-CRE Recombinase) (Cat. #6653)
AAVpro® Helper Free System (AAV6-CRE Recombinase) (Cat. #6654)
AAVpro® Helper Free System (AAV1-LacZ) (Cat. #6669)
AAVpro® Helper Free System (AAV2-LacZ) (Cat. #6655)
AAVpro® Helper Free System (AAV5-LacZ) (Cat. #6656)
AAVpro® Helper Free System (AAV6-LacZ) (Cat. #6657)
AAVpro® Helper Free System (AAV1-U6-ZsGreen1) (Cat. #6670)*
AAVpro® Helper Free System (AAV2-U6-ZsGreen1) (Cat. #6658)
AAVpro® Helper Free System (AAV5-U6-ZsGreen1) (Cat. #6659)*
AAVpro® Helper Free System (AAV6-U6-ZsGreen1) (Cat. #6660)*
AAVpro® Helper Free System (AAV1-2xU6) (Cat. #6671)*
AAVpro® Helper Free System (AAV2-2xU6) (Cat. #6661)*
AAVpro® Helper Free System (AAV5-2xU6) (Cat. #6662)*
AAVpro® Helper Free System (AAV6-2xU6) (Cat. #6663)*
pAAV-ZsGreen1 Vector (Cat. #6231)
AAVpro® Packaging Plasmid (AAV1) (Cat. #6672)
AAVpro® Packaging Plasmid (AAV2) (Cat. #6234)
AAVpro® Packaging Plasmid (AAV5) (Cat. #6664)
AAVpro® Packaging Plasmid (AAV6) (Cat. #6665)

CalPhos™ Mammalian Transfection Kit (Cat. #631312)

AAVpro® 293T Cell Line (Cat. #632273)

AAVpro® Purification Kit Maxi (All Serotypes) (Cat. #6666)
AAVpro® Purification Kit Midi (All Serotypes) (Cat. #6675)
AAVpro® Extraction Solution (Cat. #6235)
AAVpro® Concentrator (Cat. #6674)
AAVpro® Freeze-Thaw Extraction Buffer (All Serotypes) (Cat. #6679)*

AAVpro® Titration Kit (for Real-Time PCR) Ver. 2 (Cat. #6233)

* Not available in all geographic regions. Please check for availability in your area.

IX. Notes

This product includes products made by Merck Millipore, but all questions about components, including those made by Merck Millipore, should be directed to Takara Bio.