Cat. # 6232

For Research Use

TaKaRa

AAVpro® Purification Kit (AAV2)

Product Manual

v201509Da

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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.

Available AAVpro Products

AAVpro® Helper Free System (AAV2)	Cat. #6230
AAVpro® Purification Kit (AAV2)	Cat. #6232
AAVpro® Titration Kit (for Real Time PCR) Ver.2	Cat. #6233
AAVpro® Packaging Plasmid (AAV2)	Cat. #6234
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

Adeno-associated virus (AAV) is one of the smallest viruses belonging to the *Parvovirus* family of the *Dependovirus* genus. AAV is a non-enveloped virus with a single-strand DNA genome. There are more than 100 serotypes of AAV, and the host specificity and characteristics of the virus differ among serotypes. For example, serotype 2 (AAV2) has been extensively researched and is the most commonly used serotype in AAV-based research, including gene therapy.

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). When transducing genes into animals using an AAV vector, it is necessary to use highly-purified particles that do not contain any residual virus-producing cells or impurities from the culture medium. In addition, when transducing cultured cells, purification of AAV particles eliminates the effects of the aforementioned impurities.

The purity and titer of the AAV vector is an important for achieving stable and effective introduction of genes into individual animals and cultured cells. Methods such as CsCl concentration gradient ultracentrifugation and iodixanol ultracentrifugation are commonly used to purify AAV2 vectors, but such methods are time-consuming and require careful technique to obtain high yields.

The AAVpro Purification Kit (AAV2) allows for simple and fast (~4 hours) AAV2 particles purification from AAV2-producing cells.

Advantages of the Product

- High purity and high yield.
- The unique AAV particle extraction method (patent pending) eliminates time-consuming steps such as those required for the freeze-and-thaw method and the sonication method.
- All buffers needed to purify AAV2 particles from vector-producing cells are included.
- Column purification; no ultracentrifugation required.
- Reduces dsDNA contamination, even without nuclease treatment.

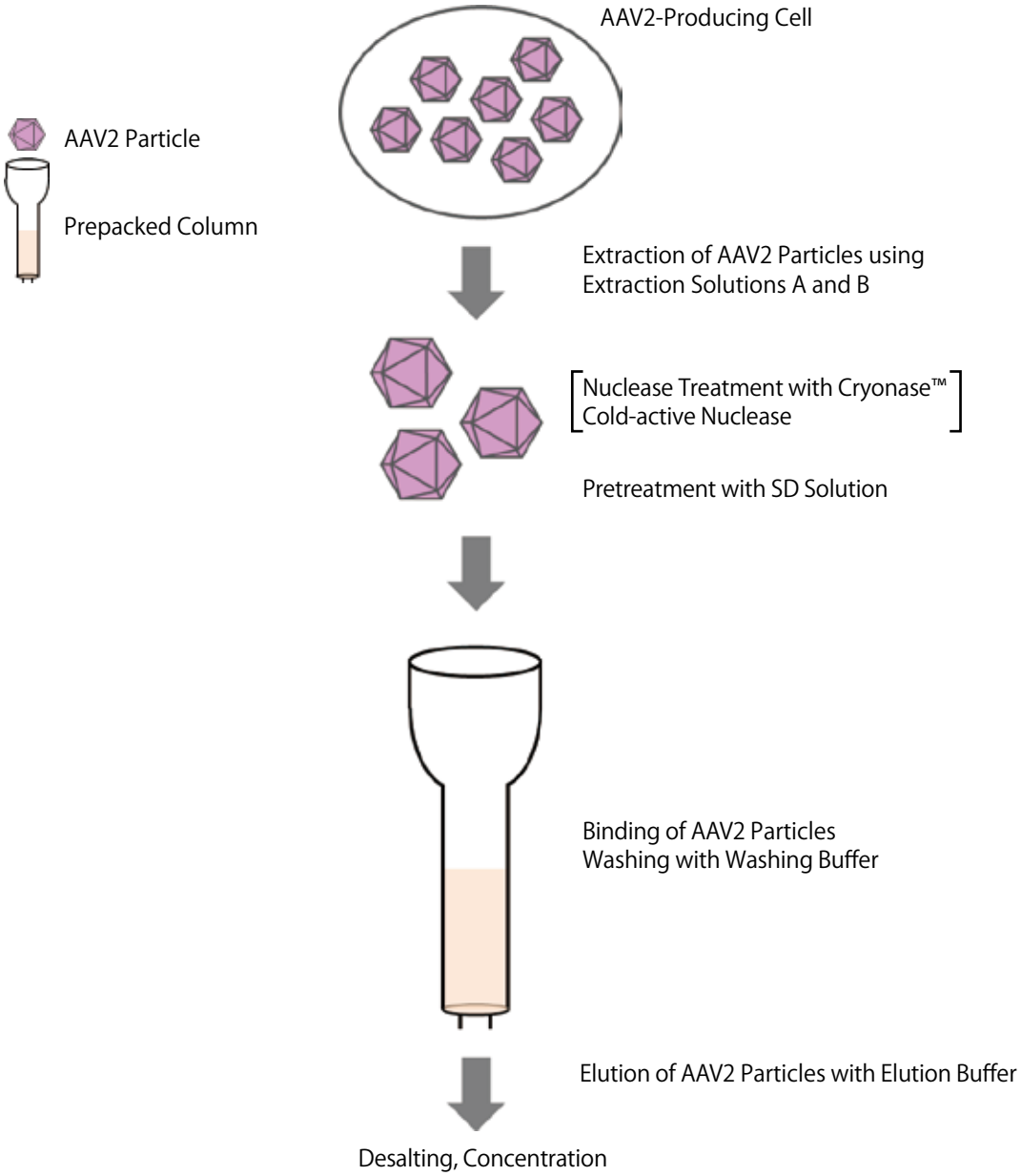


Figure 1. Overview of AAV2 Purification using the AAVpro Purification Kit (AAV2)

II. Components (2 preps)

1.	AAV Extraction Solution A	14 ml x 2
2.	AAV Extraction Solution B	2.8 ml
3.	SD Solution＊1	2.8 ml
4.	Equilibration Buffer	11 ml x 2
5.	Washing Buffer	11 ml x 2
6.	Elution Buffer	7 ml
7.	Suspension Buffer	1.2 ml
8.	Prepacked Column＊2	2
9.	Filter Device	2
10.	Collection Tube	4

＊1: White precipitate may form in the SD solution; warm at 37℃ and completely dissolve any precipitant before use.

＊2: Each Prepacked Column can purify AAV2 from up to ten T225 flasks. Purification from five T225 flasks is recommended.

III. Storage 4℃

IV. Materials Required but not Provided

[Equipment]

- General equipment necessary for cell culture
- Sterile 50-ml centrifuge tubes
- T225 flasks
- Sterile 15-ml centrifuge tubes

[Reagents]

- 0.5 M EDTA (pH 8.0)
  (e.g., EDTA Buffer Powder, pH 8.0 (Cat. #T9191))

(The following reagents are also necessary when nuclease treatment is performed)

- 1 M $MgCl_2$
- Cryonase Cold-active Nuclease (Cat. #2670A)

V. Protocol

The following protocol is for the purification of AAV2 particles from five T225 flasks. It is possible to perform the protocol below on as many as ten T225 flasks without changing the amount of buffer used.
AAVpro Helper Free System (AAV2) (Cat. #6230) is recommended for producing AAV2 vector.

V-1. Preparation of AAV2 Vector Extract

This kit contains Extraction Solution A and B to purify particles from AAV2-producing cells without the freeze-and-thaw or sonication methods that are used conventionally. These solutions make it possible to extract AAV2 vectors in an efficient and simple manner while removing host cell proteins and nucleic acids (see "VI. Experimental Examples").

1. Add 1/80 volume of 0.5 M EDTA (pH 8.0) to a culture containing AAV2-producing cells＊ and mix well. Allow to stand for 10 minutes at room temperature.

   ＊: Culture cells according to standard procedures.
2. Transfer the detached cells from the flasks to multiple sterile 50-ml centrifuge tubes.
3. Centrifuge at 1,750x *g* for 10 minutes at 4℃. Remove the supernatant, leaving approximately 5 ml. Loosen the cell pellets by tapping and collect all cells in a single sterile 50-ml centrifuge tube. Centrifuge at 1,750x *g* for 10 minutes at 4℃. Completely remove the supernatant and collect the cell pellet.
   Note: Confirm that the supernatant has been removed completely, as any residual supernatant may affect the later steps.
4. Loosen the cell pellet sufficiently by tapping or vortexing.
   Note: If the cell pellet has not been loosened completely, the efficiency of extraction may decrease. Confirm that there are no clumps of cells before moving on to the next step.
5. Add 10 ml of AAV Extraction Solution A.
6. Suspend the cells by vortexing for 15 seconds.
7. Allow the tube to stand for 5 minutes at room temperature. Then vortex for another 15 seconds.
8. Centrifuge at 2,000 - 14,000x *g* for 10 minutes at 4℃.
   Note: If the titer of the recovered AAV2 vector is low, the efficiency may be increased by repeating steps 6 - 8.
9. Collect the supernatant in a new sterile centrifuge tube and add 1 ml of AAV Extraction Solution B.
   Note 1: The mixture can be stored at -80℃ at this point. If not storing, continue promptly to step V-1-10 or V-1-11. If storing at -80℃, thaw quickly in a 37℃ water bath before proceeding.
   Note 2: The supernatant may change to a pink color after AAV Extraction Solution B is added.
   Note 3: If performing nuclease treatment, proceed with step 10N-1. If nuclease treatment will not be performed, continue with step 11.
10. Perform nuclease treatment. (Refer to "VI. Experimental Examples" for additional information.)
    10N-1. Add 1/100 volume of 1 M $MgCl_2$ solution to the supernatant mixture obtained in V-1-9.
    10N-2. Add Cryonase Cold-active Nuclease to a final concentration of 2 U/μl; incubate at 37℃ for 30 minutes.
    10N-3. Centrifuge at 2,000 - 14,000x *g* for 10 minutes at 4℃ and collect the supernatant in a new, sterile 50-ml centrifuge tube.

11. Add 1 ml of SD solution, then incubate for 30 minutes at 37℃. After, centrifuge at 2,000 - 14,000x *g* for 10 minutes at 4℃, recover the supernatant as the crude AAV2 vector solution.

    Note: White precipitates may be deposited in the SD solution, warm at 37℃ for completely dissolving them then use it.

V-2. Adsorption, Wash, and Elution of AAV2 Particles

The Prepacked Column is a gravity flow column. Before use, set up a column stand and a bottle or tube for flow-through recovery in a safety cabinet. Place the column in an upright position and confirm that resin surface is horizontal. During the purification steps, do not allow the resin to dry.

Note: Check to be no air bubble in the resin and column. If air bubble is present, resuspend the resin by pipetting to remove air then use it after sedimentation of the resin.

12. Set up the Prepacked Column. Remove the top and bottom caps and drain the excess solution inside. Add 10 ml of Equilibration Buffer and equilibrate the column.
13. Add the crude AAV2 vector solution from in step V-1-11 to the column and allow to flow through.
14. Wash the column with 10 ml of the washing buffer.
15. Set up a sterile 15 ml centrifuge tube for recovery, then elute the AAV2 Particles using 3 ml of Elution Buffer.

V-3. Desalting and Concentration of the AAV2 Particles

16. Set the Filter Device in the Collection Tube.
17. Add 500 μl of the eluted AAV2 particle solution from step V-2-15 to the Filter Device and centrifuge at 2,000x *g* for 5 minutes at 4℃. Discard the flow-through.
18. Add additional AAV2 particle solution to the Filter Device and repeat centrifugation, and discard the flow-through in the same manner as in step 17. All AAV2 particle solution may be applied to the Filter Device by repeating this process. After applying the last of the solution, centrifuge until the amount of solution in the Filter Device becomes no more than 100 μl.
19. Apply 400 μl of Equilibration Buffer to the Filter Device, centrifuge at 2,000x *g* for 5 minutes at 4℃, and discard the flow-through.

    Note: Confirm that the amount of fluid in the Filter Device is less than 100 μl. If not, repeat centrifugation.
20. Add 400 μl of Suspension Buffer to the Filter Device and pipette at least 100 times to wash the membrane of the Filter Device.
21. Recover the Suspension Buffer in a new Collection Tube, then invert the Filter Device in a new Collection Tube. Centrifuge at 1,000x *g* for 1 minute at 4℃. The solution recovered is the purified AAV2 particles.

VI. Experimental Examples

1. Performance Comparison with AAV2 Purification Kits from Other Manufacturers

AAV2 particles containing the fluorescent protein ZsGreen1 gene were purified from AAV2-producing cells, prepared with AAVpro Helper Free System (AAV2) in five T225 flasks, using this kit and two other commercially available AAV2 purification kits (Company A and Company B).

Yield of AAV2 Vector

The quantity of purified AAV2 particles obtained with each kit was determined using AAVpro Titration Kit (for Real Time PCR) Ver.2 (Cat. #6233) (Figure 2). The yield obtained using the AAVpro Purification Kit (AAV2) was ~3 times greater than that obtained with the other companies' purification kits.

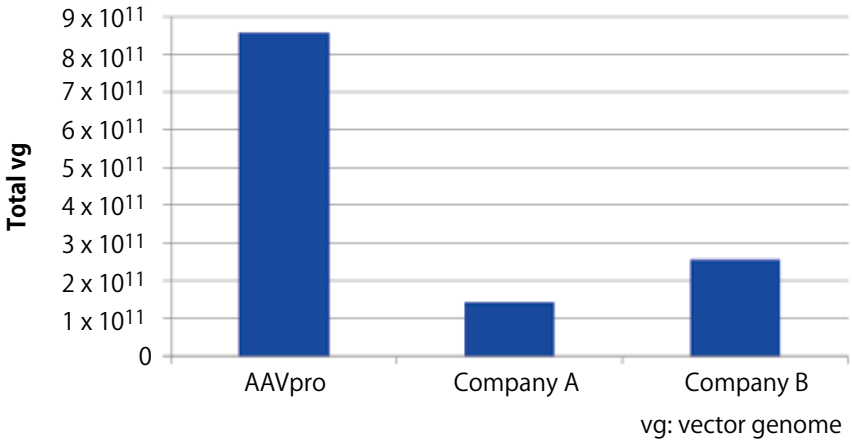
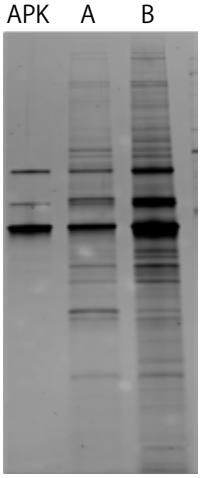


Figure 2. Yield of AAV2 Vector Purified using Various Kits

Purity

The purity of the purified AAV2 particles was evaluated by SDS-PAGE using 1 x 10^9 vector genomes (vg) per lane (Figure 3). AAV2 particles purified using the AAVpro Purification Kit (AAV2) had the highest purity; only bands derived from AAV2 proteins were observed.



APK: AAVpro Purification Kit (AAV2)
A: AAV2 purification kit, Company A
B: AAV2 purification kit, Company B

Figure 3. SDS-PAGE of the AAV2 Particles Purified using each Kit.

Infectivity of HT1080 Cells

The infectivity titer of purified AAV2 particles was evaluated (Figure 4). Infection of HT1080 cells with purified AAV2 particles containing the ZsGreen1 gene was performed at 2,500 vg/cell in all cases. Infection rate was assessed using flow cytometry at 3 days post-infection.
The results show that the AAV2 particles purified using AAVpro Purification Kit (AAV2) has the highest rate of infection when compared to particles purified using the kits from other companies.

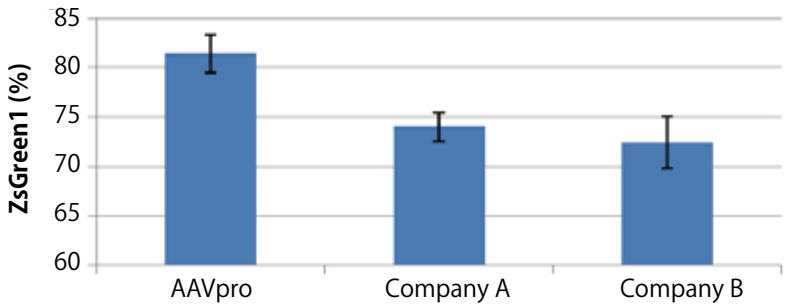


Figure 4. Infectious Titer of AAV2 Particles Purified with Various Kits

2. Residual dsDNA in AAV2 Purified with this Kit

AAV2 particle solutions obtained with this kit contain very little residual dsDNA. It is possible to further reduce residual dsDNA by treating with nuclease (step V-1-10).

AAV2 particles were purified from AAV2 vector-producing cells in five T225 flasks according to the protocol for this kit, both with and without nuclease treatment. As a comparison, the cell extract was prepared using the freeze-thaw method instead of the AAV Extraction Solutions A and B, then AAV2 vector was purified using this kit. The residual dsDNA in the purified AAV2 solutions was assessed using the intercalation method (Figure 5). The amount of residual dsDNA in the AAV2 solution purified with this kit was clearly reduced in comparison to the freeze-thaw method, even when nuclease treatment was not performed. In addition, the amount of residual dsDNA was further reduced when nuclease treatment was performed.

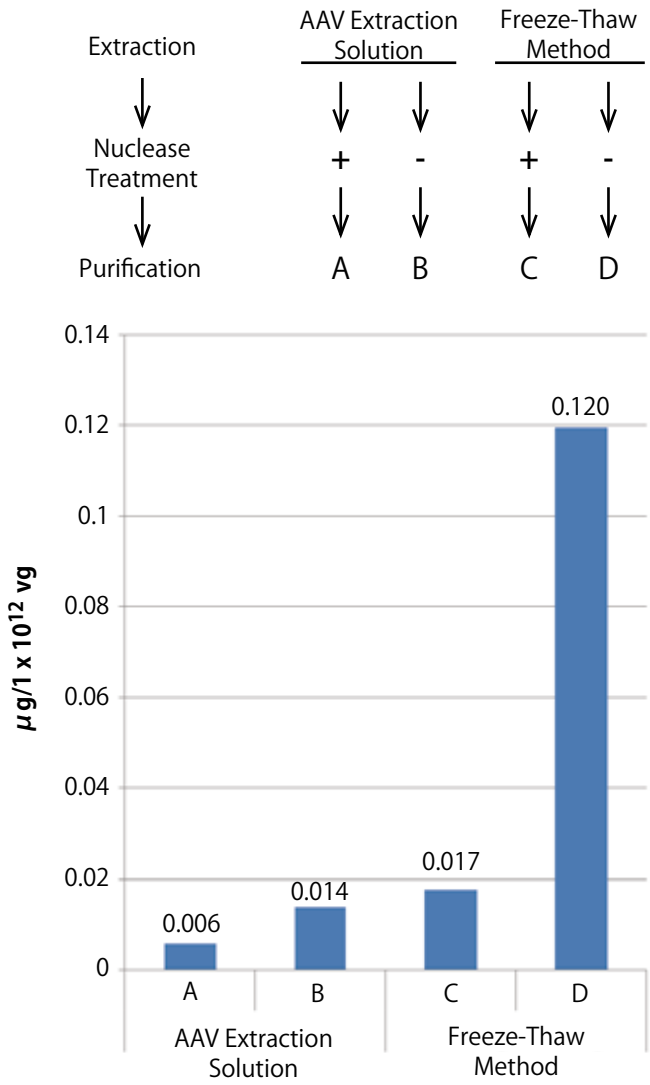
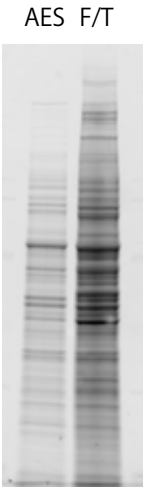


Figure 5. Amount of dsDNA in AAV2 Solutions

3. Impurities in AAV2 Particles Extracted using AAV Extraction Solution

The use of the AAV Extraction Solution A and B can eliminate impurities. Extraction of AAV2 particles from AAV2 vector-producing cells in five T225 flasks was performed using Extraction Solution A and B. AAV2 was extracted using the freeze-thaw method as a comparison. The vector genome in the AAV2 extract solutions was determined by real-time PCR. Then, 1 x 10^9 vg of AAV2 extract solution was analyzed by SDS-PAGE to evaluate the amount of protein impurity (Figure 6). In addition, residual dsDNA content was assayed using the intercalation method (Figure 7). The results indicate that the use of the AAV Extraction Solutions clearly reduced the amount of protein impurities and dsDNA in comparison with the freeze-thaw method.



AES: AAV Extraction Solution
F/T: Freeze-Thaw Method
(1 x 10^9 vg/lane)

Figure 6. SDS-PAGE of AAV2 Extraction Solutions

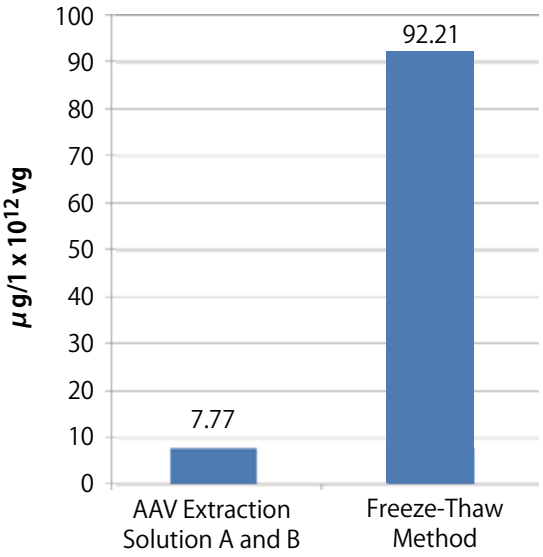


Figure 7. dsDNA in Extract Solutions

VII. References

1) Summerford, C. and Samulski, R. J. (1999) *Nat Med*. **5**:587-588.
2) Auricchio A., *et al*. (2001) *Hum Gene Ther*. **12**:71-76.

VIII. Related Products

Cryonase™ Cold-active Nuclease (Cat. #2670A)
EDTA Buffer Powder, pH 8.0 (Cat. #T9191)
AAVpro® Helper Free System (Cat. #6230)
AAVpro® Helper Free System (AAV2-CRE Recombinase) (Cat. #6652)
AAVpro® Helper Free System (AAV2-LacZ) (Cat. #6655)
AAVpro® Extraction Solution (Cat. #6235)
AAVpro® Packaging Plasmid (AAV2) (Cat. #6234)
AAVpro® Titration Kit (for Real Time PCR) Ver.2 (Cat. #6233)
pAAV-ZsGreen1 Vector (Cat. #6231)
AAVpro® Tet-One™ Inducible Expression System (AAV2) (Cat. #634310)