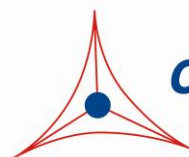

Product Manual

AAV-DJ/8 Helper Free Bicistronic Expression System (GFP)

Catalog Number

VPK-418-DJ-8 1 kit

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures



CELL BIOLABS, INC.
Creating Solutions for Life Science Research

Introduction

Adeno-associated viruses (AAVs) are derived from defective parvoviruses, which depend on essential helper functions provided by other viruses, such as adenovirus and herpes virus, for efficient viral replication and propagation. AAV has no etiologic association with any known diseases and has been successfully used to establish efficient and long-term gene expression *in vivo* in a variety of tissues without significant cellular immune responses or toxicity.

AAV has a single-stranded DNA genome which consists of approximately 4.7 kb. All characterized AAV serotypes share three key features, including two copies of AAV terminal repeats (ITRs), one *rep* region and one *cap* region. The ITRs are capable of forming T-shape secondary structure and are the only *cis* elements that are required for AAV replication, packaging, integration, and rescue. The *rep* region encodes four overlapping proteins designated as Rep78, Rep68, Rep52, and Rep40, according to the apparent molecular mass of the protein. In addition to their well-defined roles in AAV replication, Rep proteins also regulate AAV packaging and site-specific integration. The *cap* region encodes three structural proteins, VP1, VP2, and VP3. These three proteins share the same reading frame (see Figure 1).

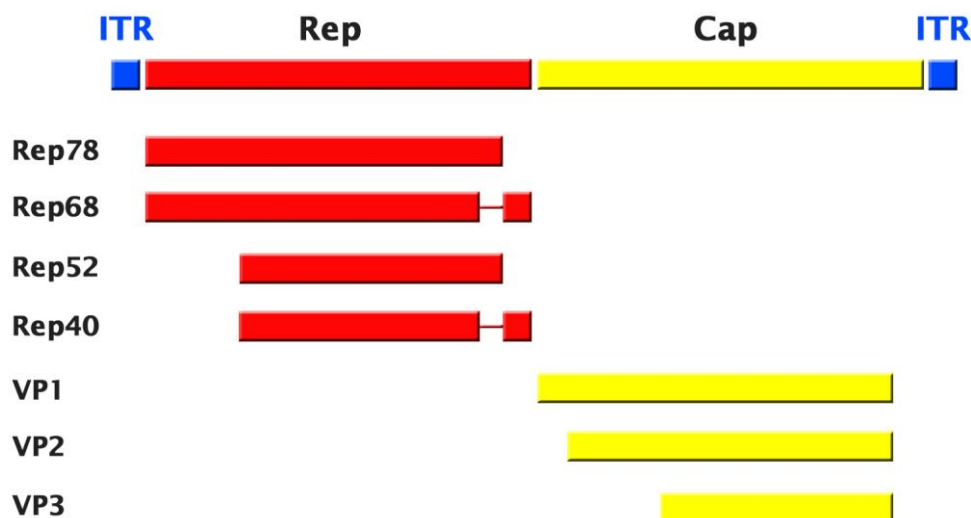


Figure 1. Schematic Map of AAV Genome.

Rep: involved in genome replication; VP1/2/3: capsid proteins.

Cell Biolabs' AAV Helper-Free System allows the production of infectious recombinant human adeno-associated virus (rAAV) virions without the use of a helper virus (Figure 2). In the AAV Helper-Free System, most of the adenovirus gene products required for the production of infective AAV particles are supplied on the plasmid pHelper (i.e. E2A, E4, and VA RNA genes) that is co-transfected into cells with human AAV vector DNA. The remaining adenoviral gene product is supplied by the 293 host cells, which stably express the adenovirus E1 gene. By eliminating the requirement for live helper virus the AAV Helper-Free System provides a safer and more convenient gene delivery system. In the AAV Helper-Free System, the *rep* and *cap* genes have been removed from the viral vector that contains AAV-2 ITRs and are supplied *in trans* on the plasmid pAAV-RC. The removal of the AAV *rep* and *cap* genes allows for insertion of a gene of interest in the viral genome. Cell Biolabs' AAV Helper-Free System can accommodate inserts of up to 3 kb (See Table 1 for detail).

Adeno-associated virus (AAV) serotypes differ broadly in transduction efficacies and tissue tropisms. DNA family shuffling technology is used to create a complex library of hybrid capsids from eight

different wild-type viruses (Figure 3). Stringent selection of AAV variants on human liver cells and with human anti-AAV antisera result in AAV-DJ (a clone named after the first two authors, see ref. 11), and AAV-DJ/8 (a heparin binding domain mutant of AAV-DJ). Recombinant AAV-DJ vectors mediate superior in vitro transduction efficacies in comparison with any other wild type serotypes. Transduction on cell types from different species and tissues, including primary human hepatocytes, melanoma cells, and embryonic stem cells, showed that AAV-DJ vectors were not only superior to all HBD-negative wild-type viruses (up to 100,000-fold better than AAV-8 or AAV-9), but also substantially better than AAV-2. (See Table 2 for detail). The heparin binding domain (HBD) plays important role for in vivo viral infection as demonstrated by comparing AAV-DJ to the DJ/8 mutant: HBD deletion alleviated the liver restriction and expanded transduction to all nonhepatic tissues, including the brain, identical to the transduction patterns of AAV-8 and AAV-9.

Recombinant adeno-associated viruses are important tools for gene delivery and expression. AAV has not been reported to cause any diseases. Together with its replication defective nature, AAV has good safety profile to be used in gene transfer in vivo, and as potential gene therapy vehicles. Recombinant AAV is capable of infecting a broad range of cell types including non-dividing cells and remaining as concatemers for long-term expression. Compared with other viral vectors such as adenovirus, AAV elicits very mild immune response in animal models.

Catalog #	Product Name	Capacity (kb)
VPK-410	pAAV-MCS	3
VPK-411	pAAV-MCS	3.9
VPK-418	pAAV-IRES-GFP	1.7

Table 1. Packaging capacity of AAV shuttle vectors.

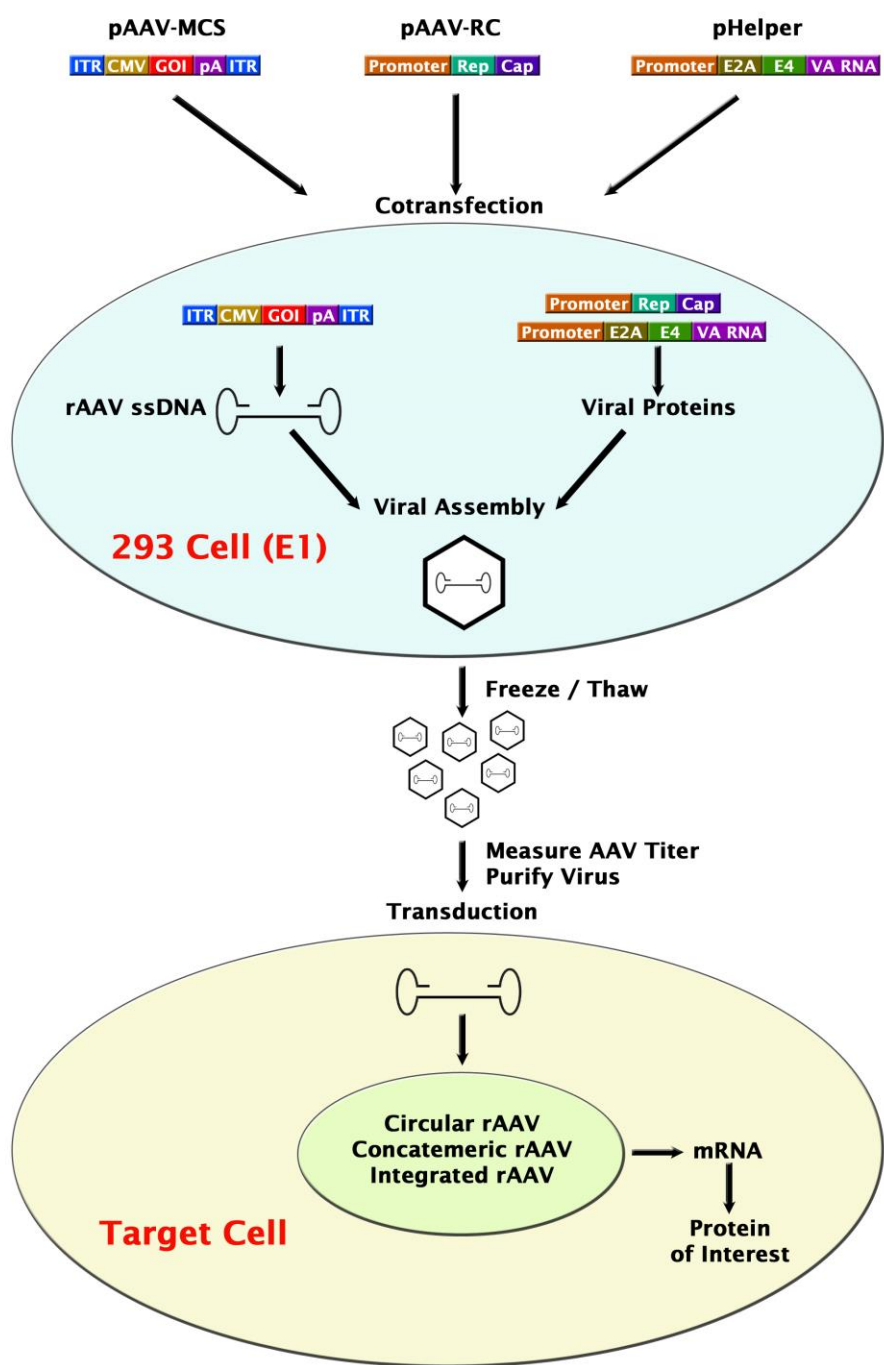


Figure 2. AAV Helper-Free System.

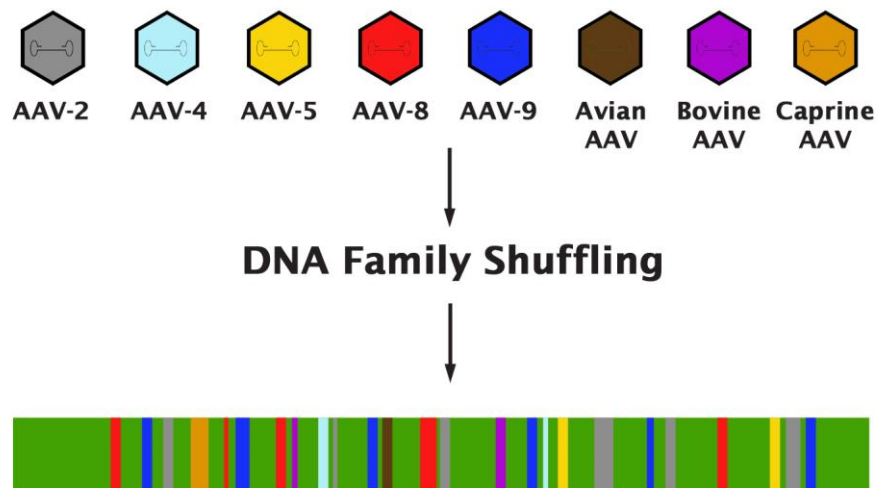


Figure 3. Generation of AAV-DJ through capsid DNA family shuffling.

Cell Line	Tissue or Cell Type	AAV-1	AAV-2	AAV-3	AAV-4	AAV-5	AAV-6	AAV-8	AAV-9	AAV-DJ	AAV-DJ/8
Huh-7	Hu Liver	13	100	2.5	0.0	0.1	10	0.7	0.0	500	0.2
HEK293	Hu Kidney	25	100	2.5	0.1	0.1	5	0.7	0.1	500	0.3
HeLa	Hu Cervix	3	100	2.0	0.1	6.7	1	0.2	0.1	667	0.2
HepG2	Hu Liver	3	100	16.7	0.3	1.7	5	0.3	ND	1250	0.5
Hep1A	Ms Liver	20	100	0.2	1.0	0.1	1	0.2	0.0	400	0.1
911	Hu Retina	17	100	11.1	0.2	0.1	17	0.1	ND	500	0.0
CHO	Hm Ovary	100	100	14.3	1.4	333	50	10.0	1.0	25000	5.0
COS	Si Kidney	33	100	33.3	3.3	5.0	14	2.0	0.5	500	0.3
MeWo	Hu Skin	10	100	20.0	0.3	6.7	10	1.0	0.2	2857	1.0
NIH3T3	Ms Fibroblasts	10	100	2.9	2.9	0.3	10	0.3	ND	500	0.1
A549	Hu Lung	14	100	20.0	ND	0.5	10	0.5	0.1	1000	0.1
HT1180	Hu Fibroblasts	20	100	10.0	0.1	0.3	33	0.5	0.1	333	0.2
Monocytes	Hu Primary Monocytes	1111	100	ND	ND	125	1429	ND	ND	100	ND
Immature DC	Hu Monocyte-derived DC	2500	100	ND	ND	222	2857	ND	ND	200	ND
Mature DC	Hu Monocyte-derived DC	2222	100	ND	ND	333	3333	ND	ND	100	ND

Table 2. In vitro relative infectivity of AAV vectors.

Related Products

1. VPK-400-DJ-8: AAV-DJ/8 Helper Free Packaging System
2. VPK-410-DJ-8: AAV-DJ/8 Helper Free Bicistronic Expression System
3. VPK-418: pAAV-IRES-GFP Expression Vector
4. AAV-100: 293AAV Cell Line
5. VPK-140: ViraBind™ AAV Purification Kit
6. VPK-141: ViraBind™ AAV Purification Mega Kit
7. VPK-145: QuickTiter™ AAV Quantitation Kit
8. AAV-200: ViraDuctin™ AAV Transduction Kit

Kit Components (shipped on blue ice)

1. pAAV-IRES-GFP Expression Vector (Part No. VPK-418): One 40 µL vial at 0.25 mg/mL.
2. pAAV-DJ/8 Vector (Part No. VPK-420-DJ-8): One 40 µL vial at 0.25 mg/mL.
3. pHelper Vector (Part No. 340202): One 40 µL vial at 0.25 mg/mL.
4. pAAV-GFP Control Vector (Part No. AAV-400): One 40 µL vial at 0.25 mg/mL.

Materials Not Supplied

1. 293 cells: we recommend 293AAV Cell Line (Cat.# AAV-100) for high titer production of AAV.
2. Cell Culture Medium
3. Transfection Reagents
4. 0.5 M EDTA

Storage

Store all components at -20°C.

Safety Considerations

Remember that you will be working with samples containing infectious virus. Follow the recommended NIH guidelines for all materials containing BSL-2 organisms. The AAV Helper-Free system is designed to minimize the chance of generating wild type AAV, but precautions should still be taken to avoid direct contact with viral supernatants.

Vector Features

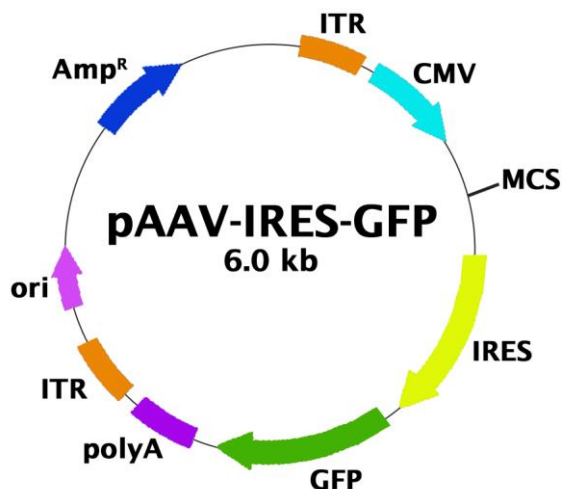


Figure 3: pAAV-IRES-GFP Expression Vector (see Appendix for more detail).

Vector Features:

1 ~ 130:	Left ITR
139 ~ 801:	CMV Promoter
809 ~ 1301:	human β -globin intron
1308 ~ 1376:	MCS
1407 ~ 1985:	IRES
1986 ~ 2705:	GFP
2717 ~ 3195:	PolyA
3235 ~ 3375:	Right ITR
4292 ~ 5152:	Ampicillin Resistance

MCS:

- Enzyme Sites: 5'- ClaI, BamHI, EcoRV, XhoI, EcoRI, XhoI -3'
- MCS Sequence:

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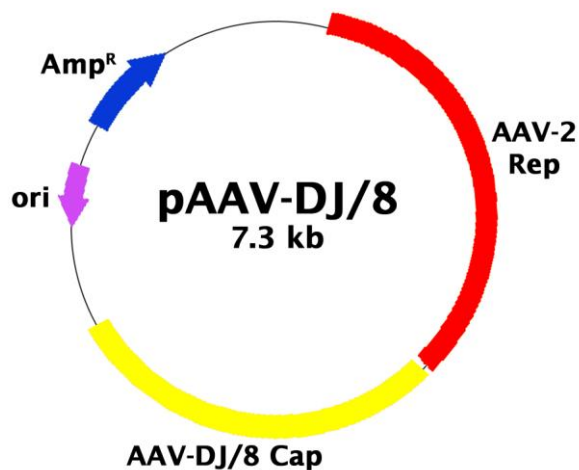


Figure 5. pAAV-DJ/8 Vector

Vector Features:

6 ~ 1871:	AAV-2 Rep
1888 ~ 4101:	AAV-DJ/8 Cap
5606 ~ 6466:	Ampicillin Resistance

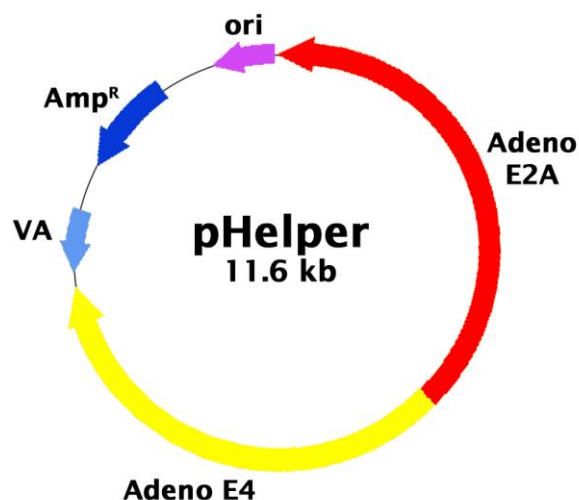


Figure 6. pHHelper Vector

Vector Features:

1 ~ 5336: Adeno E2A
 5337 ~ 8537: Adeno E4
 8535 ~ 9280: Adeno VA
 10182 ~ 11042: Ampicillin Resistance

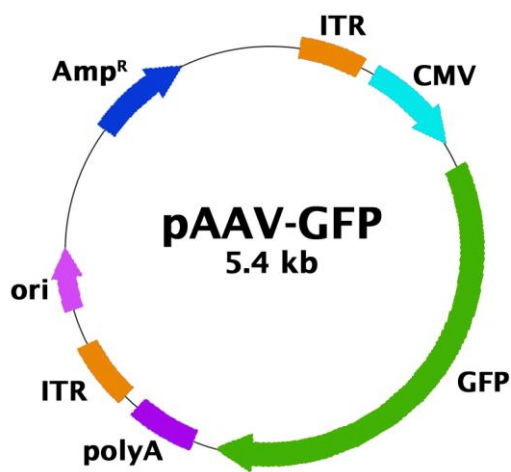


Figure 7. pAAV-GFP Vector

Vector Features:

1 ~ 130: Left ITR
 139 ~ 801: CMV Promoter
 809 ~ 1301: human β -globin intron
 1324 ~ 2064: GFP
 2123 ~ 2601: PolyA
 2635 ~ 2775: Right ITR
 3692 ~ 4552: Ampicillin Resistance

rAAV Production

1. One day before transfection, plate sufficient 293 cells or 293AAV cells (Cat. # AAV-100) to achieve 70-80% confluence on the day of transfection.
2. Cotransfect cells with pAAV Expression vector, pAAV-DJ/8 and pHHelper.
Notes:
 - We recommend the ratio of vectors at 1:1:1 (pAAV Expression Vector:pAAV-DJ/8:pHelper).
 - Calcium Phosphate transfection method is preferred for AAV production. For lipid-based transfection reagents, we only suggest FuGENE® 6 (Roche Applied Science) or Lipofectamine™ LTX (Invitrogen).
3. 48-72 hours after transfection, add 0.5 M EDTA to a final of 10 mM to the plate and incubate for 3 min at room temperature. Gently shake the culture plate several times and harvest all media, including cells, in a sterile tube.

Notes:

- *As viral production proceeds, some of the cells will round up and detach from the plate, and can be seen as floating in the medium.*
 - *Viruses are present in both intact cells and the growth medium. For more concentrated virus stock, we only recommend proceeding with cell pellet.*
4. Centrifuge the cell suspension at 1000 RPM for 5 min. Remove the supernatant and resuspend the cell pellet in desired amount of DMEM or sterile PBS.
 5. Freeze and thaw the cell suspension four times by placing it alternately in a dry ice/ethanol bath and a water bath of 37°C. Remove cell debris by centrifugation at 10,000 g for 10 min and collect the supernatant as AAV crude lysate.
 6. AAV crude lysate can be used directly or purified/concentrated if needed. For long term storage, store supernatant at -80°C in aliquots.

Post-Packaging Considerations

The quality of rAAV vector preparations is a key element in obtaining reliable and reproducible data. Purification of rAAV from crude cell lysate is usually required before transduction of your target cells. rAAV is usually quantified by genome copy (GC) number. These genome-containing particles are functional vectors that infect target cells. Besides these "full" AAV, empty viral particles are also produced. Measurement of GC rather than total particle number is thus more relevant.

1. **Concentration and purification of your rAAV:** Recombinant AAV vector can be purified by CsCl gradient ultracentrifugation, iodixanol discontinuous gradient ultracentrifugation, and high-performance liquid chromatography (HPLC).
2. **Measure titer of your rAAV:**
 - a. **Genome Copy (GC) Number:** This is an important step to ensure consistent viral transduction into your host cell. However, QPCR or dot blot of viral DNA can take as much as 1-4 days to complete. An ELISA method has been developed by using antibody that only reacts with AAV intact particles; however, this method measures all AAV particles including the ones lacking genomic DNA. Cell Biolabs' QuickTiter™ AAV Quantitation Kit (Catalog # VPK-145) does not involve cell infection; instead it specifically measures the viral nucleic acid content of purified virus. The entire procedure takes about 30 minutes for purified AAV.
 - b. **Infectious Titer:** For AAV vector containing reporter, the rAAV infectious titer can be determined using either green fluorescent protein (GFP) or LacZ as the reporter gene. For rAAV-LacZ, each blue cell after X-Gal staining represents one infectious unit (IU). For rAAV-GFP, each green cell under fluorescence microscopy represents one IU.
3. **Use transduction reagents to increase infection efficiency:** The AAV transduction process includes viral binding and entry, intracellular trafficking, nuclear transport, and viral second strand DNA synthesis. The viral second strand DNA synthesis has been shown to be the rate limiting step, which leads to inefficient transduction by AAV vectors. Cell Biolabs' ViraDuctin™ AAV Transduction Kit (Catalog # AAV-200) is designed to increase transduction efficiencies by AAV on both dividing and non-dividing cells.

References

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Appendix

pAAV-IRES-GFP Plasmid Features and Sequence

1-130:	Left ITR
139-798:	CMV Promoter
806-1298:	Human β -globin Intron
1305-1373:	MCS
1404-1982:	IRES
1983-2702:	GFP
2714-3192:	PolyA
3232-3372:	Right ITR
4289-5149:	Ampicillin Resistance

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