

AAV Helper-Free System

Instruction Manual

Catalog #240071 (AAV Helper-Free System)

#240074 (pAAV-hrGFP Control Plasmid)

#240075 (pAAV-IRES-hrGFP Vector)

Revision B

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240071-12



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Prior to use of the AAV Helper-Free System, we strongly recommend that the user become thoroughly familiar with the safety considerations concerning the production and handling of AAV and adenovirus. For a description of laboratory biosafety level criteria, consult the Centers for Disease Control Office of Health and Safety Web site <http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s3.htm>. Production of adeno-associated virus and use of AAV vectors fall within NIH Biosafety Level 2 criteria. Any vector containing potentially toxic or oncogenic inserts should be handled with BSL-2+ precautions. For more information regarding handling AAV and BSL-2+ practices, consult the UCSD Vector Development Lab material data safety sheet at <http://medicine.ucsd.edu/gt/AAV.html> and the UCSD Environmental Health and Safety Web site <http://www-ehs.ucsd.edu/ADENO.HTM>.

AAV Helper-Free System

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AAV Helper-Free System

MATERIALS PROVIDED

AAV Helper-Free System (Catalog #240071) Materials Provided	Concentration	Quantity
pAAV-MCS vector	1 µg/µl in TE buffer	10 µg
pCMV-MCS vector	1 µg/µl in TE buffer	10 µg
pAAV-LacZ vector	1 µg/µl in TE buffer	10 µg
pAAV-RC plasmid (sufficient for two transfections)	1 µg/µl in TE buffer	20 µg
pHelper plasmid (sufficient for two transfections)	1 µg/µl in TE buffer	20 µg
AAV-293 cells (Catalog #240073)	—	1 × 10 ⁶ cells
AAV-HT1080 cells (Catalog #240109)	—	1 × 10 ⁶ cells

pAAV-hrGFP (Catalog #240074) Materials Provided	Concentration	Quantity
pAAV-hrGFP control plasmid	1 µg/µl in TE buffer	20 µg

pAAV-IRES-hrGFP (Catalog #240075) Materials Provided	Concentration	Quantity
pAAV-IRES-hrGFP vector	1 µg/µl in TE buffer	10 µg

STORAGE CONDITIONS

AAV Vectors: –20°C

AAV-293 and AAV-HT1080 Cells: Place in liquid nitrogen immediately upon arrival.

ADDITIONAL MATERIALS REQUIRED

Alkaline phosphatase, molecular biology grade

Competent *E. coli* cells (*endA*, *recA*, e.g. XL10-Gold ultracompetent cells, Stratagene Catalog #200314)

Growth media for AAV-293/HT1080 cells[§]

Reagents for transfection of AAV-293 cells (e.g. CaCl₂ plus 2×HBS[§])

In Situ β-Galactosidase Staining Kit (Stratagene Catalog #200384)

Camptothecin[§] (Sigma-Aldrich, Inc.)

[§] See *Preparation of Media and Reagents*.

Revision B

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IRES Sequence

Use of the translation enhancer of the pIRES-hrGFP-1a and pIRES-hrGFP-2a vectors is covered by U.S. Patent No. 4,937,190 and is limited to use solely for research purposes. Any other use of the translation enhancer of the pIRES-hrGFP-1a and pIRES-hrGFP-2a vectors requires a license from WARF. WARF can be reached at P.O. Box 7365 Madison, WI 53707-7365.

INTRODUCTION

Adeno-associated viruses (AAVs) are replication-deficient parvoviruses, which have traditionally required co-infection with a helper adenovirus or herpes virus for productive infection. The AAV Helper-Free System allows the production of infectious recombinant human adeno-associated virus-2 (AAV-2) virions without the use of a helper virus. The AAV Helper-Free System takes advantage of the identification of the specific adenovirus gene products that mediate AAV replication and the demonstration that these gene products can be introduced into the host cell by transfection.¹ 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-2 vector DNA. The remaining adenoviral gene product is supplied by the AAV-293 host cells, which stably express the adenovirus E1 gene.² The AAV Helper-Free System includes AAV-293 cells, which are HEK293-derived cells with improved adeno-associated virus production capabilities. By eliminating the requirement for live helper virus the AAV Helper-Free System provides a safer, purer and more convenient alternative to retroviral and adenoviral gene delivery systems.

The wild-type AAV-2 genome consists of the viral *rep* and *cap* genes (encoding replication and capsid genes, respectively), flanked by inverted terminal repeats (ITRs) that contain all the *cis*-acting elements necessary for replication and packaging. 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. The AAV Helper-Free System can accommodate inserts of up to 3 kb.

In traditional viral delivery systems, regeneration of wild-type virus by recombination is a major concern. In the AAV Helper-Free system, the AAV-2 ITR-containing plasmids (pAAV-MCS, pAAV-LacZ and pAAV-hrGFP and pAAV-IRES-hrGFP) do not share any regions of homology with the *rep/cap*-gene containing plasmid (pAAV-RC), preventing the production of wild-type AAV-2 through recombination. To ensure that this lack of homology is maintained, it is important to use all of the components provided with the AAV Helper-Free system together. In particular, only pAAV-RC should be used as the source of *rep* and *cap* for co-transfection with the ITR-containing vectors.

The AAV Helper-Free System eliminates the requirement for wild-type adenovirus co-infection from both AAV vector production and AAV stock titering steps, making this system entirely helper virus-free. Conventional AAV vector titering methods involve co-infection by wild-type adenovirus and the AAV vector stock. This co-infection titering method is complicated by the fact that the optimal MOI for adenovirus co-infection varies among cell types and often requires optimization. Our AAV Helper-Free system features a novel adenovirus-free titering method, which removes the requirement for co-infection with adenovirus but gives results comparable to adenovirus co-infection protocols.

Recombinant adeno-associated viruses are important tools for gene delivery and expression (for a review, see reference 3). The AAV Helper-Free system features versatility in host range, high titer virus production capacity, and long-term gene transfer potential. AAV is capable of infecting a broad range of cell types and infection is not dependent on active host cell division. In addition, since the AAV Helper-Free System does not involve any wild-type virus production, host immune responses are minimized, allowing gene-delivery to immunocompetent hosts. The ability to produce recombinant virus at high titer is an important consideration for mammalian cell gene delivery strategies. The AAV Helper-Free System produces recombinant viral titers of $\geq 10^7$ viral particles/ml of primary virus stock. (It is reasonable to expect higher titers after concentration of the virus. Titers as high as $\geq 10^{12}$ viral particles/ml after concentration have been published. See references 4, 5, and 6 for virus stock concentration and purification methods.)

AAV-2 has proven especially valuable for long-term gene expression due to the ability of AAV-2 to replicate epichromosomally under replication-permissive conditions. Slowly dividing or non-dividing cells can maintain the epichromosomal AAV-2 genome over time, and gene expression will be stable while the AAV-2 genome is maintained.⁷ Rapidly dividing cells will lose epichromosomal genome after cell replication and will, therefore, lose gene expression. Viral integration into the genome can occur, however, integration events are rare. The frequency of integration events may increase if an extremely high multiplicity of infection is used or if the cell is infected in the presence of adenoviral replicase.

Advantages of Recombinant Adeno-Associated Virus for Gene Delivery and Expression

Superior biosafety rating

AAV-2 is a naturally defective virus, requiring provision of several factors in *trans* for productive infection and has not been associated with any human disease. In the AAV Helper-Free System, the AAV-2 ITR sequences and *rep/cap* genes are present on separate plasmids that lack homology, preventing production of recombinant wild-type virus. These features give the AAV Helper-Free System a superior biosafety rating among gene delivery and expression vectors of viral origin.

Broad range of infectivity

Adeno-associated viruses infect a broad range of mammalian cells and have been used successfully to express human and non-human proteins. In contrast with other vectors of viral origin, adeno-associated virus vectors have proven competent for gene expression in immunocompetent hosts.

High titer

Recombinant AAV-2 can be produced at high titers of $\geq 10^7$ viral particles/ml with this protocol. Titers as high as $\geq 10^{12}$ viral particles/ml after concentration of the primary virus stock have been published. See references 4, 5, and 6 for virus stock concentration and purification methods.

Infection does not require an actively dividing host cell

Recombinant adeno-associated virus can infect both dividing and non-dividing cells.

Expressed human proteins are properly folded and modified

Because the AAV Helper-Free System delivers genes to human host cell lines, human proteins expressed using this system have the correct post-translational modification and folding.

Long-term gene transfer potential

Recombinant AAV-2 can be maintained in the human host cell, creating the potential for long-term gene transfer. In all cell populations, the viral genome typically remains epichromosomal, often forming concatemers. These concatemers are stable in slowly dividing or non-dividing cells, leading to long-term gene transfer and expression in these cell populations. However, in rapidly dividing cell populations, the epichromosomal AAV genome is lost. The virus can integrate into the host genome, leading to long-term gene expression in dividing cells, but this is a rare event. The likelihood of integration occurring increases if an extremely high multiplicity of infection (MOI) of AAV is used or if infection of the target cell occurs in the presence of adenoviral replicase, potentially supplied by the use of wild-type adenovirus. However, use of wild-type adenovirus to increase integration events reduces the biosafety of the AAV system.

OVERVIEW OF THE AAV HELPER-FREE SYSTEM

A schematic overview of the production of recombinant AAV particles using the AAV Helper-Free System is shown in Figure 1. The first step is cloning the gene of interest into an appropriate plasmid vector. For most applications, the DNA of interest is cloned into one of the ITR/MCS-containing vectors (pAAV-MCS or pAAV-IRES-hrGFP). The inverted terminal repeat (ITR) sequences present in these vectors provide all of the *cis*-acting elements necessary for AAV-2 replication and packaging.

In some instances insertion of certain sequences directly into an ITR-containing vector may lead to rearrangements due to homologous recombination between the ITRs in *E. coli*. In such cases, the gene of interest should first be cloned into the shuttle vector pCMV-MCS. Once this construct has been verified, the *Not* I fragment containing the expression cassette is subcloned into one of the pAAV ITR-containing vectors, such as pAAV-MCS, that has been digested with *Not* I.

The recombinant expression plasmid is co-transfected into the AAV-293 cells with pHelper (carrying adenovirus-derived genes) and pAAV-RC (carrying AAV-2 replication and capsid genes), which together supply all of the *trans*-acting factors required for AAV replication and packaging in the AAV-293 cells. Recombinant AAV-2 viral particles are prepared from infected AAV-293 cells and may then be used to infect a variety of mammalian cells.

Upon infection of the host cell, the single-stranded virus must become double-stranded in order for gene expression to occur. The virus relies on cellular replication factors for synthesis of the complementary strand.^{8, 9} This conversion is a limiting step in recombinant gene expression and can be accelerated using adenovirus superinfection or etoposides like camptothecin or sodium butyrate. However, agents to accelerate gene expression are toxic to the target cells and can kill target cells if left on the cells. The use of etoposides is therefore only recommended for short-term use or in obtaining viral titers. Typically, the AAV genome will form high molecular weight concatemers which are responsible for stable gene expression in cells.

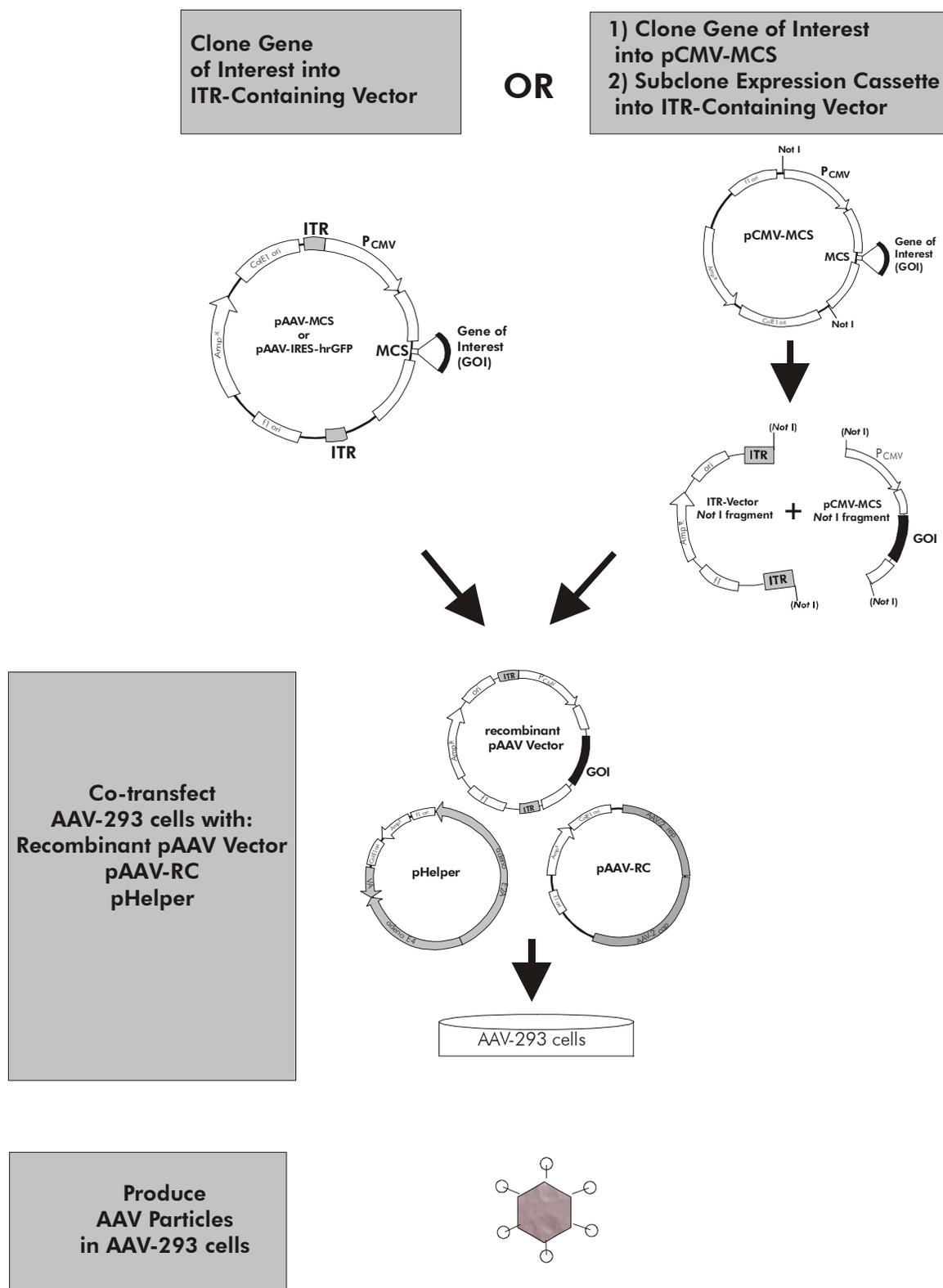


FIGURE 1 Production of recombinant AAV particles

Vector Features

The pAAV-MCS and the pAAV-IRES-hrGFP (Catalog #240075, Figure 2) vectors contain the CMV promoter and other elements for high-level gene expression in mammalian cells when a gene of interest is cloned into the multiple cloning site (MCS). Both vectors also contain AAV-2 inverted terminal repeats (ITRs), which direct viral replication and packaging.

Besides these shared features, the pAAV-IRES-hrGFP vector contains a dicistronic expression cassette in which the humanized recombinant green fluorescent protein (hrGFP) from a novel marine organism is expressed as a second open reading frame that is translated from the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES). An important benefit gained from expressing the gene of interest from pAAV-IRES-hrGFP is that hrGFP fluorescence may be used to measure the titer of the recombinant virus stock directly. In addition, since both proteins are translated from the same transcript, hrGFP expression may be used to ascertain the infection efficiency for the desired target cell type and also serves as a useful expression marker for the inserted gene of interest. pAAV-IRES-hrGFP features the 3× FLAG[®] sequence at the C-terminus of the MCS. Foreign genes cloned in-frame with the tag may be detected or purified using an anti-FLAG antibody. This feature is very useful when antibodies against the protein encoded by the transgene are unavailable. Insert size is limited in these vectors by size constraints on AAV-2 packaging. The markerless pAAV-MCS vector is recommended for inserts approaching 3 kb in size, while pAAV-IRES-hrGFP can accommodate inserts ≤1.7 kb.

Note *If the gene of interest is cloned into pAAV-IRES-hrGFP, the titer of the recombinant viral stock may be measured directly. However, because the reporter is expressed at relatively low levels due to the IRES sequence preceding the hrGFP ORF, we have measured a 10-fold lower viral titer of recombinant pAAV-IRES-hrGFP-AAV when compared to pAAV-hrGFP-AAV viral titer.*

pCMV-MCS (Figure 3) may be used as a shuttle vector for inserts that are unstable in the ITR-containing vectors. This vector provides the CMV promoter and other elements for high-level gene expression in mammalian cells but lacks ITR sequences. The expression cassette is flanked by *Not* I restriction sites that are used to subclone the expression cassette containing the gene of interest into an ITR-containing vector. Any of the pAAV ITR-containing vectors (pAAV-MCS, pAAV-IRES-hrGFP, pAAV-LacZ, or pAAV-hrGFP) may be used as the acceptor vector for this subcloning step.

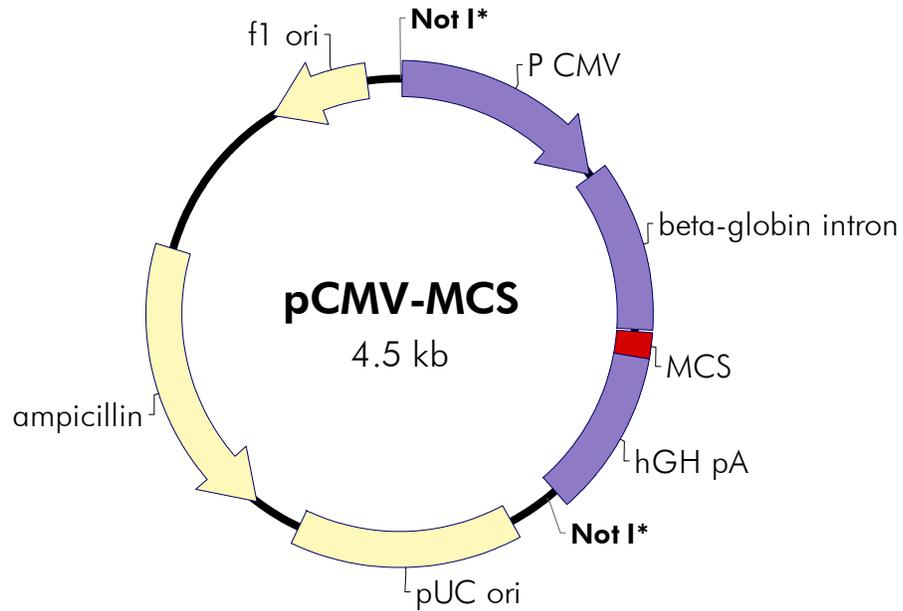
Vector Features continued

pAAV-LacZ (Figure 4) and pAAV-hrGFP (Catalog #240074, Figure 5) serve as both a reporter plasmid for monitoring the AAV-293 producer cell transfection efficiency at the viral packaging step and as a control for monitoring viral production and should be used as an indirect measurement of the recombinant viral titer. In these control vectors, the ITR sequences flank a cassette that directs expression of *LacZ* or hrGFP, respectively, from the CMV promoter. pAAV-hrGFP can be used to qualitatively assess the transfection efficiency of the producer cell line and to determine the viral titer using fluorescence microscopy or by fluorescence activated cell sorting (FACS).

The pAAV-RC plasmid (Figure 6) contains the AAV-2 *rep* and *cap* genes, encoding replication proteins and viral capsid structural proteins, respectively. Establishing the proper expression levels of *rep* and *cap* gene products is an important step in achieving high-titer virus production.¹⁰ pAAV-RC directs Rep and Cap expression from two different promoters, achieving optimal expression levels for each set of gene products.

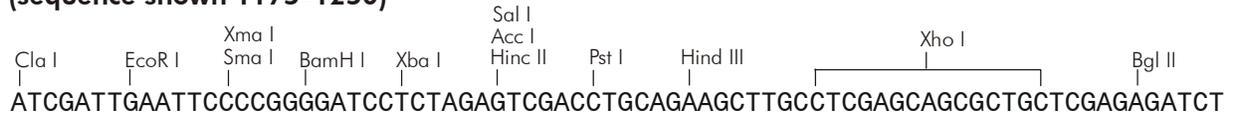
The pHelper plasmid (Figure 7) contains the subset of adenovirus genes, VA, E2A and E4, which are necessary for high-titer AAV production in the AAV-293 cells. Adenovirus proteins E1A and E1B, which are also required for AAV-2 production, are stably expressed in the AAV-293 cells.²

pCMV-MCS Vector



*Non-unique sites used to release the expression cassette for subcloning into an ITR-containing vector

pCMV-MCS Multiple Cloning Site Region (sequence shown 1175–1250)

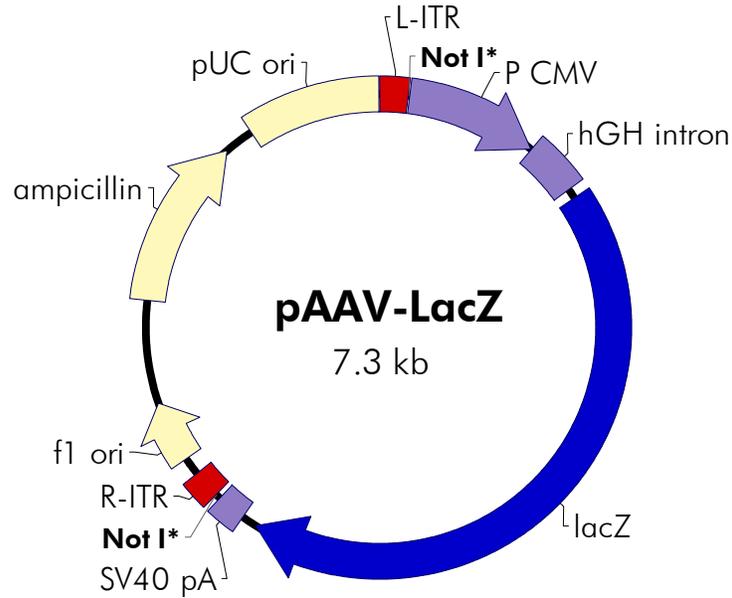


†The two Xho I restriction sites are adjacent in the MCS and are suitable for use in cloning

Feature	Nucleotide Position
CMV promoter	9–667
β-globin intron	675–1167
multiple cloning site	1175–1250
human growth hormone (hGH) polyA signal	1246–1732
pUC origin of replication	1893–2560
ampicillin resistance (<i>bla</i>) ORF	2708–3568
f1 origin of ss-DNA replication	4087–4393
Not I cleavage sites for subcloning into ITR-containing plasmid	1, 1761

FIGURE 3 The pCMV-MCS cloning vector

pAAV-LacZ Plasmid

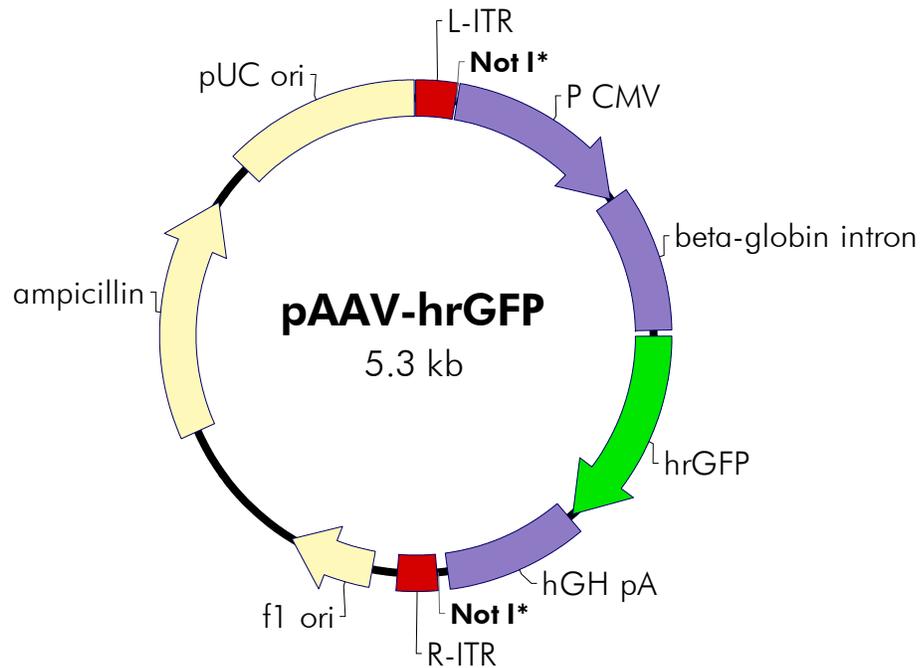


*Sites used to release and subclone expression cassettes

Feature	Nucleotide Position
left AAV-2 inverted terminal repeat (ITR)	1–141
CMV promoter	150–812
human growth hormone (hGH) intron	818–1089
β-galactosidase (<i>lacZ</i>) ORF	1141–4281
SV40 polyA signal	4363–4508
right AAV-2 inverted terminal repeat (ITR)	4535–4675
f1 origin of ss-DNA replication	4767–5073
ampicillin resistance (<i>bla</i>) ORF	5592–6449
pUC origin of replication	6600–7267
Not I cleavage sites for subcloning from pCMV-MCS	142, 4527

FIGURE 4 The pAAV-LacZ plasmid

pAAV-hrGFP Plasmid

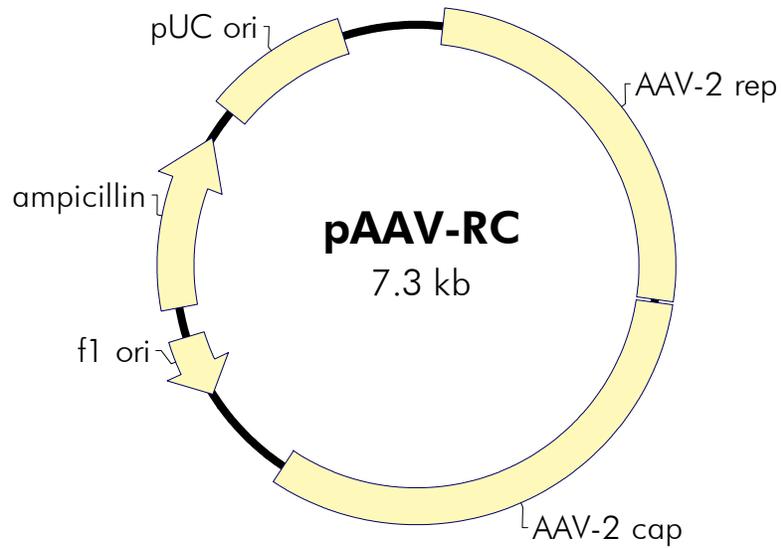


*Sites used to release and subclone expression cassettes

Feature	Nucleotide Position
left AAV-2 inverted terminal repeat (ITR)	1-141
CMV promoter	150-812
β -globin intron	820-1312
hrGFP ORF	1335-2051
human growth hormone (hGH) polyA signal	2070-2548
right AAV-2 inverted terminal repeat (ITR)	2588-2728
f1 origin of ss-DNA replication	2820-3126
ampicillin resistance (<i>bla</i>) ORF	3645-4499
pUC origin of replication	4653-5320
Not I cleavage sites for subcloning from pCMV-MCS	143, 2581

FIGURE 5 The pAAV-hrGFP plasmid

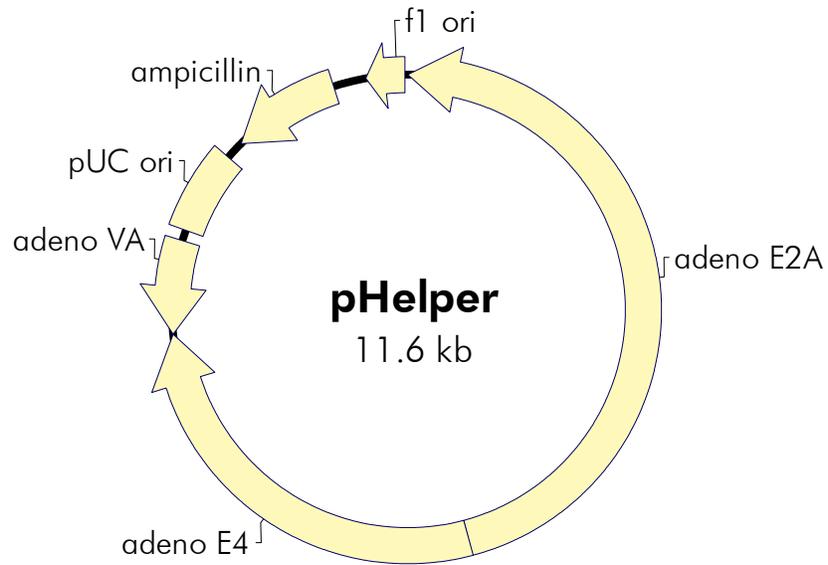
pAAV-RC Plasmid



Feature	Nucleotide Position
AAV-2 <i>rep</i> gene	131–1996
AAV-2 <i>cap</i> gene	2013–4346
f1 origin of ss-DNA replication	4838–5143
ampicillin resistance (<i>bla</i>) ORF	5292–6149
pUC origin of replication	6300–6967

FIGURE 6 The pAAV-RC plasmid

pHelper Plasmid



Feature	Nucleotide Position
adenovirus E2A gene	1–5336
adenovirus E4 gene	5337–8537
adenovirus VA gene	8538–9280
pUC origin of replication	9367–10034
ampicillin resistance (<i>bla</i>) ORF	10182–11042
f1 origin of ss-DNA replication	11305–11600

FIGURE 7 The pHelper plasmid

RECOMMENDED PRIMER SEQUENCES

Verification of Cloning

Sequences and binding sites of primers suitable for PCR amplification and/or sequencing applications with pAAV-MCS, pAAV-IRES-hrGFP and pCMV-MCS are shown in the table below.

Primer Identity	Sequence (5'–3')	Primer Binding Sites		
		pAAV-MCS	pAAV-IRES-hrGFP	pCMV-MCS
Forward (β-globin)	ATTCTGAGTCCAAGCTAGGC	1177–1196	1192–1211	1048–1067
Reverse (hGH polyA)	TAGAAGGACACCTAGTCAGA	1487–1506	2993–3012	1358–1377
T3 Forward	ATTAACCCTCACTAAAGGGA	none	1326–1345	none
T7 Reverse	TAATACGACTCACTATAGGG	none	2861–2880	none

Verification of Subcloning

The *Not* I subcloning junctions in the AAV Helper-Free System vectors are adjacent to the AAV-2 ITR sequences which are refractory to DNA sequencing. See *Subcloning from pCMV-MCS into an ITR-Containing Vector* for a restriction-digestion based alternative for examination of the integrity of the ITR sequences at the *Not* I subcloning junctions. For PCR verification of *Not* I subcloning, we recommend using one of the vector-specific primers listed in the table below in combination with an appropriate insert-specific primer.

Primer Identity	Sequence (5'–3')	Primer Binding Sites			
		pAAV-MCS	pAAV-LacZ	pAAV-IRES-hrGFP	pAAV-hrGFP
Forward (left ITR)	CCTCTGACTTGAGCGTCGAT	4517–4536	7153–7172	6023–6042	5207–5226
Reverse (right ITR)	TACTATGGTTGCTTTGACGT	2094–2113	4730–4749	3600–3619	2784–2803

RECOMMENDED BACTERIAL STRAIN FOR CLONING AND DNA PROPAGATION

XL10-Gold ultracompetent cells* (Catalog #200314) are recommended to amplify the AAV Helper-Free System plasmids and for the cloning and subcloning steps of recombinant AAV production. This strain is both endonuclease deficient (*endA1*) and recombination deficient (*recA*). The *endA1* mutation greatly improves the quality of plasmid miniprep DNA, and the *recA* mutation helps ensure insert stability.

In the following table, the genes indicated in italics signify that the bacterium carries a mutant allele. The genes present on the F' episome represent the wild-type bacterial alleles.

Host strain	Genotype
XL10-Gold ultracompetent cells	Tet ^R $\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173$ <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte</i> [F' <i>proAB lacI^qZ</i> Δ M15 Tn10 (Tet ^R) Amy Cam ^R]

* U.S. Patent Nos. 6,706,525, 5,512,486, 5,707,841, and equivalent foreign patents.

GENERATING AAV RECOMBINANT PLASMIDS

Cloning Considerations

The gene of interest to be inserted into the pAAV-MCS, or pCMV-MCS vectors must include both initiation and stop codons. The gene of interest to be inserted into the pAAV-IRES-hrGFP vector must contain an initiation codon but not stop codon since the stop codon is located 3' of the FLAG sequence. If the FLAG sequence is translated, the gene of interest and the FLAG sequence must be in the same reading frame. We recommend including a Kozak initiation sequence¹¹ for optimal translation of the gene of interest. A complete Kozak sequence includes CCACCATGG, although CCATGG, or the core initiator ATG, is sufficient.

The recommended maximum insert size for cloning into pAAV-MCS or pCMV-MCS is 3 kb, and for pAAV-IRES-hrGFP is 1.7kb. These size limitations are based upon size constraints on AAV-2 packaging.

Genes of interest cloned into the pCMV-MCS vector must later be excised by *Not* I digestion for subcloning into an ITR-containing vector. Ensure that the gene of interest does not contain *Not* I restriction sites. Perform site-directed mutagenesis to remove any *Not* I sites from the gene of interest before proceeding to the cloning steps.

Notes *We recommend performing site-directed mutagenesis to remove Not I sites from the gene of interest using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene Catalog #200524).*

Prior to preparing viral particles containing the recombinant AAV expression vector, expression of the gene of interest may be confirmed by transiently-transfecting host cells using a high efficiency transfection method followed by immunodetection methods such as immunocytochemistry or Western blot analysis. Transfection efficiency of the host cells can be determined by transfecting the host cells with the pAAV-hrGFP vector and detecting expression of hrGFP. If the protein of interest is not detected in the transiently-transfected cells, it is unlikely that it will be detected later in infected cells.

Refer to Figures 2–4 for multiple-cloning-site sequences and to www.stratagene.com for the complete nucleotide sequence and restriction maps of the AAV Helper-Free System vectors. The DNA sequence at the cloning junctions has been verified for each of the AAV Helper-Free System vectors. The remainder of the vector sequences were compiled from existing data.

Cloning the Gene of Interest in pAAV-MCS, pAAV-IRES-hrGFP, or pCMV-MCS

1. Clone the gene of interest into the desired vector using appropriate site(s) in the MCS. For general DNA cloning protocols, see reference 12.
2. Confirm the presence of the insert by restriction digestion, by PCR using one vector-specific primer plus one gene-specific primer, or by sequence analysis.
3. Determine the nucleotide sequence of the insert.
3. If the gene of interest was cloned directly into pAAV-MCS or pAAV-IRES-hrGFP, prepare the recombinant AAV expression plasmid DNA in sufficient quantities for subsequent transfection experiments. Each transfection requires 10 µg of the recombinant pAAV plasmid DNA plus 10 µg each of pAAV-RC and pHelper. This DNA must be of high quality and purity (i.e. prepared using standard cesium chloride density gradient centrifugation or affinity column purification that produces DNA of equivalent quality). Resuspend purified plasmid DNA in TE buffer, pH 7.5 (See *Preparation of Media and Reagents*). Proceed to *Preparation of Primary AAV Stocks*.

If the gene of interest was cloned into pCMV-MCS, proceed to *Subcloning from pCMV-MCS into an ITR-Containing Vector*, below.

Subcloning from pCMV-MCS into an ITR-Containing Vector

1. Digest pAAV-MCS (or another pAAV ITR-containing vector) with *Not* I restriction enzyme, according to the enzyme manufacturer's instructions. Confirm complete digestion by agarose gel electrophoresis with an aliquot of digested DNA. *Not* I digestion of pAAV-MCS should produce a 2.9 kb vector fragment containing the AAV-2 ITRs and a 1.8 kb fragment containing the MCS.
2. Once complete digestion with *Not* I is confirmed, treat the *Not* I-digested pAAV-MCS DNA with alkaline phosphatase for 30 minutes at 37°C.
3. Digest the recombinant pCMV-MCS plasmid containing the gene of interest with *Not* I. Digestion of recombinant pCMV-MCS with *Not* I should produce a 2.7 kb vector fragment and a fragment of variable length (1.8 kb plus the length of the gene of interest) containing the CMV expression cassette.

4. Gel-purify the dephosphorylated 2.9 kb vector fragment from pAAV-MCS and the *Not* I fragment containing the gene of interest from pCMV-MCS using agarose gel electrophoresis. Include samples of uncut plasmids in adjacent lanes for comparison. Ensure that the gel is run long enough to visualize good separation between uncut DNA and each of the DNA fragment bands. Recover the DNA from the gel using the StrataPrep DNA gel extraction kit (Catalog # 400766) or using another gel extraction method of choice.
5. Ligate the purified vector and expression cassette fragments with DNA ligase. For DNA ligation protocols, see reference 12. Transform an aliquot of the ligation mixture into XL10-Gold chemically competent cells (other *endA*⁻, *recA*⁻ competent *E. coli* cells may also be used).
6. Identify recombinant clones by restriction digestion or PCR analysis.

Notes *The Not I subcloning step in the AAV Helper-Free System is bi-directional. Both orientations of the expression cassette in the AAV expression vector are expected to produce equivalent levels of gene expression and of recombinant virus production.*

The Not I cloning junctions in the recombinant AAV expression plasmid are adjacent to the AAV-2 ITR sequences. The sequences of the left and right ITRs are identical to each other, contain repetitive DNA, and are GC-rich, making these elements refractory to DNA sequence analysis. The gross integrity of these elements can be analyzed by releasing the ITRs by digestion with Not I plus Pst I. The Not I-Pst I fragments released from the pAAV-MCS plasmid should be 126 bp and 145 bp. The presence of the expected ITR fragments can be confirmed by polyacrylamide gel electrophoresis.

7. Prepare the recombinant AAV expression plasmid DNA in sufficient quantities for subsequent transfection experiments. Each transfection will require 10 µg of the recombinant pAAV plasmid DNA plus 10 µg each of pAAV-RC and pHelper. This DNA must be of high quality and purity for transfection of mammalian cells (i.e. prepared using standard cesium chloride density gradient centrifugation or affinity column purification that produces DNA of equivalent quality). Resuspend purified plasmid DNA in TE buffer, pH 7.5.

AAV-293 CELL CULTURE GUIDELINES

Description of the AAV-293 Cells

We recommend preparing adeno-associated recombinant virus stocks using the AAV-293 cell line provided. The AAV-293 cells provided are derived from the commonly used HEK293 cell line, but produce higher viral titers. HEK293 cells are human embryonic kidney cells that have been transformed by sheared adenovirus type 5 DNA.² AAV-293 cells, like HEK293 cells, produce E1 *in trans*, allowing the production of infectious virus particles when cells are transfected with E1-deleted adenovirus vectors or when co-transfected with the three AAV Helper-Free System plasmids (an ITR-containing plasmid, pAAV-RC, and pHelper).

Notes *It is necessary to use the AAV-293 cells provided to produce viral particles using the AAV Helper-Free System. Substitution with other cell lines will not work, since replication of the AAV-2 vector requires the adenovirus E1 gene product that is stably expressed in the HEK293-derived AAV-293 cells.*

All procedures must be performed using sterile technique in a laminar flow hood. For general information on mammalian cell culture and sterile technique, see reference 13.

AAV-293 cells do not adhere well to tissue culture dishes and have a tendency to clump. When exchanging solutions, gently pipette down the side of the dish and not directly onto the cells to prevent disruption of the cell monolayer.

It is important to maintain the AAV-293 cells at $\leq 50\%$ confluence to ensure the integrity of the stock, maintaining the higher titer production phenotype.

It is important to prepare a liquid nitrogen stock of early passage cell aliquots for long-range experiments.

Establishing AAV-293 Cultures from Frozen Cells

1. Place 10 ml of DMEM growth medium (see *Preparation of Media and Reagents*) in a 15-ml conical tube.
2. Thaw the frozen cryovial of cells within 1–2 minutes by gentle agitation in a 37°C water bath. Remove the cryovial from the water bath and decontaminate the cryovial by wiping the surface of the vial with 70% (v/v) ethanol (at room temperature).
3. Transfer the thawed cell suspension to the conical tube containing 10 ml of growth medium.
4. Collect the cells by centrifugation at $200 \times g$ for 3 minutes at room temperature. Remove the growth medium by aspiration.

5. Resuspend the cells in the conical tube in 5 ml of fresh growth medium by gently pipetting up and down.
6. Transfer the 5 ml of cell suspension to a 75-cm² tissue culture flask containing 10 ml of fresh growth medium. Place the cells in a 37°C incubator at 5% CO₂.
7. Monitor cell density daily. Cells should be passaged when the culture reaches 50% confluence. Proceed to either *Passaging of AAV-293 Cells* or *Preparation of an AAV-293 Cell Liquid Nitrogen Stock*.

Passaging of AAV-293 Cells

Notes *The following protocol passages cells from 75-cm² tissue culture flasks.*

Cells should be passaged when the cell monolayer reaches 50% confluence. If cell confluence exceeds 70%, AAV-293 cells may lose the increased virus production phenotype.

1. Prewarm the DMEM growth medium and trypsin-EDTA solution[§] to 37°C in a water bath.
2. Remove the growth medium by aspiration. Wash the cells once with 10 ml of phosphate-buffered saline (PBS).[§]
3. Trypsinize the cells for 1–3 minutes in 5 ml of trypsin-EDTA solution.

Note *Incubate the cells in the trypsin-EDTA solution for the minimum time required to release adherent cells from the flask. This process may be monitored using an inverted microscope. Excess trypsinization may damage or kill the cells.*

4. Dilute the cells with 5 ml of growth medium to inactivate the trypsin.
5. Transfer 2 ml of the cell suspension to each of five 175-cm² tissue culture flasks, each containing 28 ml of fresh growth medium. Place the cells in a 37°C incubator at 5% CO₂. Monitor cell density daily. Cell confluence should be maintained at 50%.

[§] See *Preparation of Media and Reagents*.

Preparation of an AAV-293 Cell Liquid Nitrogen Stock

Notes *The following protocol prepares frozen stocks from 175-cm² tissue culture flasks.*

It is important to maintain cells that are propagated to establish a liquid nitrogen stock at $\leq 50\%$ confluence to ensure the integrity of the stock, maintaining the higher titer production phenotype.

Feed the cells one day prior to preparing liquid nitrogen stocks to improve viability.

1. Prewarm the DMEM growth medium, trypsin-EDTA solution, and freezing medium to 37°C in a water bath.
2. Collect cells from a healthy, log-phase culture. Remove the culture medium by aspiration. Wash the cells with 10 ml of PBS. Trypsinize the cells for 1–3 minutes in 10 ml of trypsin-EDTA solution.

Note *Incubate the cells in the trypsin-EDTA solution for the minimum time required to release adherent cells from the flask. This process may be monitored using an inverted microscope. Excess trypsinization may damage or kill the cells.*

3. Dilute the cells with 10 ml of growth medium to inactivate the trypsin. Transfer the cell suspension into a 50-ml conical tube. Count the cells present in an aliquot of the resuspension using a hemocytometer.
4. Collect the cells by centrifugation at 200 × g for 10 minutes at room temperature. Remove the growth medium by aspiration.
5. Resuspend the cell suspension to 1 × 10⁶ cells/ml in freezing medium (see *Preparation of Media and Reagents*; do not include antibiotics), then dispense 1-ml aliquots of the suspension into 2-ml cryovials.
6. Freeze the cell aliquots gradually by placing the vials in a Styrofoam® container and then placing the container in a –80°C freezer overnight.
7. Transfer the vials of frozen cells to liquid nitrogen for long-term storage.

PREPARATION OF PRIMARY AAV STOCKS

Safety Considerations

See *Preprotocol Safety Considerations* for important information regarding safety considerations for the use of AAV and adenovirus vectors. The safety guidelines presented are not intended to replace the BSL 2+ safety procedures already in place at your facility. The information set forth in this manual is intended as an additional resource and to supplement existing protocols in your laboratory.

The steps performed in this and the following sections, *Preparation of Primary Adeno-Associated Viral Stocks* and *pAAV-LacZ and pAAV-hrGFP Control Applications*, need to be carried out under sterile conditions in a laminar flow hood designated for use with virus. For handling AAV or adenovirus-containing solutions, use disposable pipets or pipettors with filter tips to prevent the transfer of contaminated aerosols.

Preparing the AAV-293 Cells

Plate the AAV-293 cells at 3×10^6 cells per 100-mm tissue culture plate in 10 ml of DMEM growth medium 48 hours prior to transfection.

Note *To achieve high titers, it is important that the AAV-293 cells are healthy and plated at optimal density. Cells should be passaged at 50% confluency. It is thus prudent to initially prepare a large number of frozen vials of the cells while they are at a low passage and healthy. Care should be taken to avoid clumping of the cells during passaging and plating for transfection. Cells may be grown to higher confluency prior to transfection with the AAV plasmids.*

Transfecting the AAV-293 Cells

Although a variety of transfection protocols may be successfully used with these vectors, the following calcium phosphate-based protocol is recommended. This protocol consistently results in the production of titers $\geq 10^7$ viral particles/ml when transfecting the AAV-293 cells.

Notes *Do not allow the transfection mixture prepared in this section to sit before it is added to the cells. The large aggregates that form after prolonged incubation are inhibitory to uptake.*

The use of lipid-based transfection reagents is not recommended.

1. Inspect the host cells that were split two days before; they should be approximately 70–80% confluent.

Note *Since there are variations of cell growth rate and methods of determining cell number, we suggest seeding the cells at several different concentrations (e.g., 2×10^6 , 3×10^6 , and 4×10^6 per 100-mm plate). Select the plate that is approximately 70–80% confluent and evenly distributed.*

2. Remove the three plasmids to be co-transfected (the recombinant pAAV expression plasmid or control plasmid, pAAV-RC, and pHelper) from storage at -20°C . Adjust the concentration of each plasmid to 1 mg/ml in TE buffer, pH 7.5.
3. Pipet 10 μl of each of the three plasmid DNA solutions (10 μg of each plasmid) into a 15-ml conical tube. Add 1 ml of 0.3 M CaCl_2 and mix gently.
4. Pipet 1 ml of $2 \times$ HBS (see *Preparation of Media and Reagents*) into a second 15-ml conical tube. Add the 1.03-ml DNA/ CaCl_2 mixture (see above) dropwise. Mix gently by inversion or by repeated pipetting.
5. **Immediately** apply the DNA/ CaCl_2 /HBS suspension to the plate of cells in a dropwise fashion, swirling gently to distribute the DNA suspension evenly in the medium.
6. Return the tissue culture plate to the 37°C incubator for 6 hours.

Note *We strongly suggest performing a viral production negative control by substituting one or all three of the plasmid(s) in the transfection mixture with TE buffer (of the same volume).*

7. At the end of the incubation period, remove the medium from the plate and replace it with 10 ml of fresh DMEM growth medium.
8. Return the plate to the 37°C incubator for an additional 66–72 hours.

Monitoring Virus Production

It is possible to monitor the progress of AAV particle production in the culture by observing phenotypic changes to the AAV-293 cell culture. For easy comparison, prepare a viral production negative control culture (i.e., no-DNA transfection) for simultaneous observation. The most obvious sign of viral production is a color change in the medium from red to orange or yellow (compare to negative control). As viral production proceeds, some of the cells will round up and detach from the plate, and can be seen floating in the medium. Microscope photographs show AAV-producing versus non-AAV-producing AAV-293 cells in Figure 8. Generally, three days post-transfection is the optimal time to prepare AAV stocks.

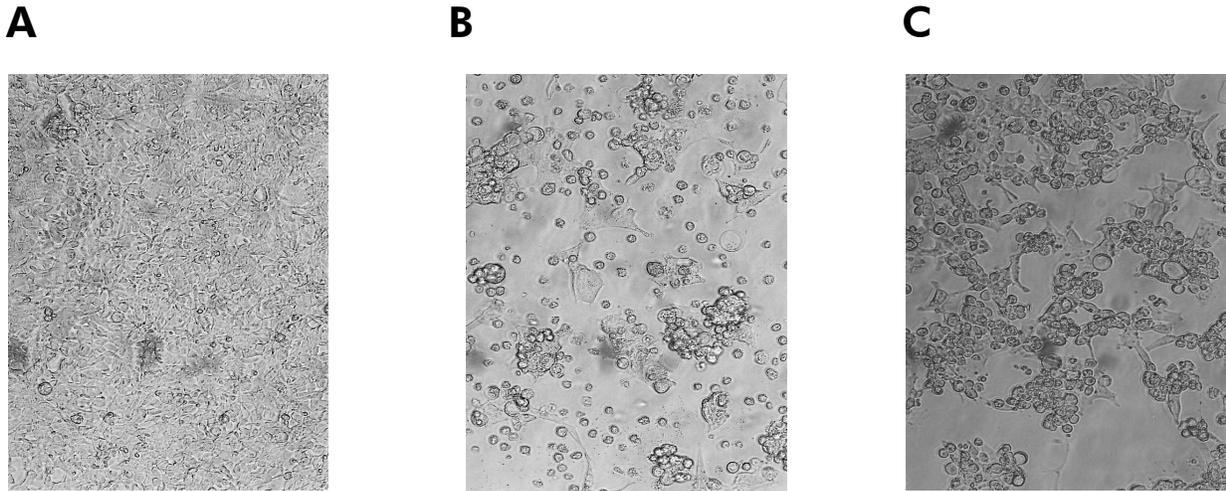


FIGURE 8 AAV particle production by the AAV-293 producer cells.

A AAV-293 cell morphology after performing the transfection protocol above with no DNA (virus production negative control). The photograph was taken at 100X magnification three days post-treatment.

B AAV-293 cell morphology after performing the transfection protocol above with pAAV-hrGFP, pAAV-RC, and pHelper. The photograph was taken at 100X magnification three days post-transfection without the removal of media and floating cells.

C AAV-293 cell morphology after performing the transfection protocol above with pAAV-LacZ, pAAV-RC, and pHelper. The photograph was taken at 200X magnification three days post-transfection following the removal of media and floating cells.

Preparing Viral Stocks

Note *Viral particles are present in both intact cells and the growth medium. Preparation of the viral stock from the combined suspension of cells plus growth medium results in the greatest yield of virus. If a more concentrated virus stock is required, see references 1, 4, 5, and 6 for virus stock concentration and purification protocols.*

1. Prepare a dry ice-ethanol bath and a 37°C water bath.
2. Transfer the transfected cells plus DMEM growth medium to a 15-ml conical tube. To collect the cells from the plate, scrape the cells into the pool of growth medium with a cell lifter, while holding the plate at an angle.
3. Subject the cell suspension to four rounds of freeze/thaw by alternating the tubes between the dry ice-ethanol bath and the 37°C water bath, vortexing briefly after each thaw.

Note *Each freeze and each thaw will require approximately 10 minutes' incubation time.*

4. Collect cellular debris by centrifugation at 10,000 × g for 10 minutes at room temperature.
5. Transfer the supernatant (primary virus stock) to a fresh tube. Viral stocks can be stored for more than one year at –80°C.
6. Viral titer may be determined indirectly for particles derived from markerless vectors using either of the control plasmids, pAAV-LacZ or pAAV-hrGFP, or directly for recombinant pAAV-IRES-hrGFP-derived viral particles. See *pAAV-LacZ and pAAV-hrGFP Control Applications* for viral titer measurement protocols. For alternative viral titer measurement protocols using quantitative PCR or slot blot methods, see references 14, 15, and 16.

AAV-HT1080 CELL CULTURE GUIDELINES (FOR TITERING)

Description of the AAV-HT1080 Cells

We recommend titrating adeno-associated recombinant virus stocks using the AAV-HT1080 cells, a human fibrosarcoma cell line. We have found the AAV-HT1080 cells to be more permissive to AAV infection than other cell lines, such as 293 cells, and thus able to give more accurate viral titers. In addition, viral titer measurement protocols using AAV-HT1080 cells have been optimized and are provided in *pAAV-LacZ* and *pAAV-hrGFP Control Applications*.

Notes *All procedures must be performed using sterile technique in a laminar flow hood. For general information on mammalian cell culture and sterile technique, see reference 13.*

It is important to prepare a liquid nitrogen stock of early passage cell aliquots for long-range experiments.

Establishing AAV-HT1080 Cultures from Frozen Cells

1. Place 10 ml of DMEM growth medium in a 15-ml conical tube.
2. Thaw the frozen cryovial of cells within 1–2 minutes by gentle agitation in a 37°C water bath. Remove the cryovial from the water bath and decontaminate the cryovial by wiping the surface of the vial with 70% (v/v) ethanol (at room temperature).
3. Transfer the thawed cell suspension to the conical tube containing 10 ml of growth medium.
4. Collect the cells by centrifugation at $200 \times g$ for 3 minutes at room temperature. Remove the growth medium by aspiration.
5. Resuspend the cells in the conical tube in 5 ml of fresh growth medium by gently pipetting up and down.
6. Transfer the 5 ml of cell suspension to a 75-cm² tissue culture flask containing 10 ml of fresh growth medium. Place the cells in a 37°C incubator at 5% CO₂.
7. Monitor cell density daily. Cells should be passaged when the culture reaches 80% confluence. Proceed to either *Passaging of AAV-HT1080 Cells* or *Preparation of an AAV-HT1080 Cell Liquid Nitrogen Stock*.

Passaging of AAV-HT1080 Cells

Note *The following protocol passages cells from 75-cm² tissue culture flasks.*

1. Prewarm the DMEM growth medium and trypsin-EDTA solution to 37°C in a water bath.
2. Remove the growth medium by aspiration. Wash the cells once with 10 ml of PBS.
3. Trypsinize the cells for 1–3 minutes in 5 ml of trypsin-EDTA solution.

Note *Incubate the cells in the trypsin-EDTA solution for the minimum time required to release adherent cells from the flask. This process may be monitored using an inverted microscope. Excess trypsinization may damage or kill the cells.*

4. Dilute the cells with 5 ml of growth medium to inactivate the trypsin.
5. Transfer 2 ml of the cell suspension to each of five 175-cm² tissue culture flasks, each containing 28 ml of fresh growth medium. Place the cells in a 37°C incubator at 5% CO₂. Monitor cell density daily. Cell confluence should be maintained at <80%.

Preparation of an AAV-HT1080 Cell Liquid Nitrogen Stock

Notes *The following protocol prepares frozen stocks from 175-cm² tissue culture flasks.*

Feed the cells one day prior to preparing liquid nitrogen stocks to improve viability.

1. Prewarm the DMEM growth medium, trypsin-EDTA solution, and freezing medium to 37°C in a water bath.
2. Collect cells from a healthy, log-phase culture (approximately 80% confluent). Remove the culture medium by aspiration. Wash the cells with 10 ml of PBS. Trypsinize the cells for 1–3 minutes in 10 ml of trypsin-EDTA solution.

Note *Incubate the cells in the trypsin-EDTA solution for the minimum time required to release adherent cells from the flask. This process may be monitored using an inverted microscope. Excess trypsinization may damage or kill the cells.*

3. Dilute the cells with 10 ml of growth medium to inactivate the trypsin. Transfer the cell suspension into a 50-ml conical tube. Count the cells present in an aliquot of the resuspension using a hemocytometer.
4. Collect the cells by centrifugation at 200 × g for 10 minutes at room temperature. Remove the growth medium by aspiration.
5. Resuspend the cell suspension to 1 × 10⁶ cells/ml in freezing medium (do not include antibiotics), then dispense 1-ml aliquots of the suspension into 2-ml cryovials.
6. Freeze the cell aliquots gradually by placing the vials in a Styrofoam container and then placing the container in a –80°C freezer overnight.
7. Transfer the vials of frozen cells to liquid nitrogen for long-term storage.

PAAV-LACZ AND PAAV-HRGFP CONTROL APPLICATIONS

Perform the *Preparation of Primary AAV Stock* protocols using the pAAV-LacZ control plasmid in parallel with the recombinant plasmid containing the gene of interest to monitor your success at various points in the procedure. Cell staining can be performed on cells containing pAAV-LacZ-derived virus to (1) confirm that the AAV-293 transfection was successful (2) estimate titer of stocks produced from transfection, and (3) test the ability of AAV vectors to infect a potential target cell. To detect the presence of LacZ, the cells are stained with X-gal. We recommend the In Situ β -Galactosidase Staining Kit (Stratagene Catalog #200384). Other in situ X-gal staining procedures will also work.

As an alternative, the plasmid pAAV-hrGFP (Catalog #240074), containing the coding sequence for hrGFP, can be used for titer determination by fluorescence activated cell sorting (FACS) analysis. Fluorescence microscopy is not recommended for titer determination due to a potentially low signal intensity and background fluorescence. This control plasmid may also be used for consistent qualitative assessment of AAV-293 producer cell transfection efficiency by fluorescence microscopy.

Note *If the gene of interest was cloned into pAAV-IRES-hrGFP, the titer of the recombinant viral stock may be measured directly using the viral titer measurement protocols provided in Viral Titer Measurement of pAAV-hrGFP AAV (Adenovirus-Free) or Viral Titer Measurement using Adenovirus Co-infection (Alternate Protocol).*

For alternative viral titer measurement protocols using quantitative PCR or slot blot methods, see references 14–16.

Transfection Control

Two days post-transfection with pAAV-LacZ, AAV-293 cells can be stained with X-gal to evaluate the success of the transfection. Follow the protocol provided with the In Situ β -Galactosidase Staining Kit, or the X-gal staining protocol of choice. Likewise, co-transfection with the pAAV-hrGFP control results in the observation of bright green fluorescence when cells are viewed using a fluorescence microscope with fluorescein filters. (See *Detection of hrGFP* for more information.)

Viral Titer Measurement of Recombinant pAAV-LacZ-AAV (Adenovirus-Free)

Use the following protocol to measure the titer of the pAAV-LacZ primary AAV stock, prepared as described in *Preparation of Primary AAV Stock*.

Preparing Permissive Cells

Note *This protocol has been optimized using the AAV-HT1080 cells provided. Other cell lines may be used for titer measurement, but the preparation of permissive cells using different cell lines will require optimization of the types and concentrations of chemical agents included to prepare permissive medium. See references 7 and 17 for agents that increase AAV transduction in certain cell lines.*

1. Plate HT1080 cells at a density of 3×10^5 per well in 1 ml of DMEM growth medium in 12-well tissue culture plates. Ensure that cells are spread evenly in wells for accurate titer determination.
2. Incubate overnight at 37°C. The cells should be ~80% confluent before proceeding. If necessary, prolong incubation to increase cell density.
3. Add 0.2 ml of AAV permissive growth medium,[§] per well, without removing the original medium (for a final concentration of 0.8 μM camptothecin in the medium). Mix well by swirling, then return the plates to 37°C incubator for 4 hours.
4. Aspirate the medium.

Viral Stock Dilution and Application

1. Dilute the primary viral stock 100-fold. From this 100-fold dilution, perform 5-fold serial dilutions in 2.5-ml volumes over a dilution series from 2×10^{-3} to 8×10^{-5} in L-DMEM.[§]

Note *The dilution series suggested above is for primary, unconcentrated viral stock. If a concentrated viral stock is used, adjust the dilution series accordingly.*

2. Add 0.5 ml of each dilution to separate wells of the 12-well plates. Perform the titer in triplicate, adding 0.5 ml of each dilution to each of three wells. In addition, include a no viral stock as a negative titer control.
3. Incubate at 37°C for 1–2 hours. Swirl plates gently at 30-minute intervals during the incubation.
4. Add 0.5 ml of pre-warmed H-DMEM[§] per well, then incubate at 37°C for 40–48 hours.

[§] See *Preparation of Media and Reagents*.

Detection of Infected Cells

1. Aspirate the growth medium.
2. Fix and stain the cells using the In Situ β -Galactosidase Staining Kit, or the X-gal staining protocol of choice.
3. Count the blue-stained cells in wells with appropriate cell densities and calculate the viral particles (number of stained cells) per ml stock. Intensity of cell staining will be variable among infected cells. Be sure to count faintly as well as intensely stained cells.

Viral Titer Measurement of Recombinant pAAV-hrGFP-AAV (Adenovirus-Free)

Since the camptothecin in the AAV permissive growth medium treatment used in the pAAV-LacZ viral titer protocol increases the autofluorescence of the AAV-HT1080 cells, we recommend using the following protocol to measure the titer of the pAAV-hrGFP primary AAV stock.

Note *If the gene of interest was cloned into pAAV-IRES-hrGFP, the titer of the recombinant viral stock may be measured directly using the protocol provided below. However, because the reporter is expressed at relatively low levels due to the IRES sequence preceding the hrGFP ORF, the pAAV-IRES-hrGFP AAV titer determined may be approximately ten-fold lower than the actual viral titer.*

Preparing Permissive Cells

Note *This protocol has been optimized using the AAV-HT1080 cells provided. Protocols using other cell lines will require optimization.*

1. Plate AAV-HT1080 cells at a density of 3×10^5 per well in 2 ml of DMEM growth medium in 6-well tissue culture plates. Ensure that cells are spread evenly in wells for accurate titer determination.
2. Incubate overnight at 37°C. The cells should be ~50% confluent before proceeding.

Viral Stock Dilution and Application

1. Dilute the primary viral stock 10-fold. From this 10-fold dilution, perform 5-fold serial dilutions in 5-ml volumes over a dilution series from 2×10^{-2} to 8×10^{-4} .

Note *The dilution series suggested above is for primary, unconcentrated viral stock. If a concentrated viral stock is used, adjust the dilution series accordingly.*

2. Add 1 ml of each dilution to separate wells of the 6-well plates. Perform the titer in triplicate, adding 1 ml of each dilution to each of three wells. Also include a no viral stock negative control.
3. Incubate at 37°C for 1–2 hours. Swirl plates gently at 30-minute intervals during the incubation.
4. Add 1 ml of pre-warmed H-DMEM per well, then incubate at 37°C for 40–48 hours.

Detection of Infected Cells

Detection of pAAV-hrGFP AAV infected cells can be done by FACS. See *Detection of hrGFP* for additional information.

Viral Titer Measurement using Adenovirus Co-infection (Alternate Protocol)

Note *The protocol provided here uses infective adenovirus to allow AAV replication. See Preprotocol Safety Considerations for information on safety considerations concerning the handling and production of adenovirus. See the adenovirus-free viral titer measurement protocols above for alternative protocols that do not require the use of infective adenovirus.*

Preparing Cells

1. Plate cells in 24-well plates at ~60% confluence in 0.5 ml of DMEM growth medium per well. Ensure that cells are spread evenly in the wells for accurate titer determination.

Note *A variety of mammalian cell lines may be used in this protocol. For HeLa cells, plate at a density of $\sim 1 \times 10^5$ cells per well.*

2. Incubate plates at 37°C overnight.

Co-infection Viral Stock Dilution and Application

1. Dilute the AAV viral stock 100-fold. From this 100-fold dilution, perform 10-fold serial dilutions in 2-ml volumes over a dilution series from 10^{-3} to 10^{-7} in DMEM growth medium. This volume of each dilution is sufficient to titer the viral stock in triplicate.
2. Add an appropriate amount of wild-type adenovirus to the AAV stock dilutions for a final MOI of 50 adenovirus per cell ($\sim 2 \times 10^7$ adenovirus particles per 2 ml AAV stock dilution to infect $\sim 3 \times 10^5$ cells in a total of three wells).
3. Aspirate medium from wells to be infected, then add 0.5-ml of each dilution to separate wells of the 24-well plates. (Perform the titer in triplicate, adding 0.5 ml of each dilution to each of three wells).
4. Incubate plates at 37°C for 20 hours.

Detection of Infected Cells

1. Aspirate the growth medium.
2. Fix and stain the cells using the In Situ β -Galactosidase Staining Kit, or the X-gal staining protocol of choice.

3. Count the blue-stained cells in wells with appropriate cell densities and calculate the viral particles (number of stained cells) per ml stock. Intensity of cell staining will be variable among infected cells. Be sure to count faintly- as well as intensely-stained cells.

Note *This protocol is also generally applicable for titer determination using the pAAV-hrGFP control or when using pAAV-IRES-hrGFP as the expression vector. One day following infection, transduced HeLa cells may be analyzed by FACS, or alternatively, titer may be determined using fluorescence microscopy by calculating the fraction of fluorescent cells in microscopic fields. See Detection of hrGFP for additional information.*

DETECTION OF HRGFP

In most cases, mammalian host cell lines transfected with pAAV-hrGFP or pAAV-IRES-hrGFP should show expression of hrGFP 24–72 hours after transfection. Fluorescing cells growing in tissue culture dishes can be observed using an inverted fluorescence microscope. Fluorescence of populations of harvested cells can also be measured using FACS analysis or fluorometer assays. The table below lists excitation and emission spectra peaks for hrGFP as compared to the widely used EGFP.

Specifications for hrGFP and EGFP Excitation and Emission Spectra

GFP Form ^a	Excitation/Emission Spectra Maxima (nm)
hrGFP	500/506
EGFP	488/509 ^b

^a Both forms of GFP compared in this table have been codon-optimized for maximum expression in human cells.

^b The emission spectrum for EGFP also shows a shoulder at 540 nm.

Note *Filter sets compatible with the detection of hrGFP and EGFP are sold by Omega Optical, Inc. (Phone: 802 254 2690, or see www.omegafilters.com):*

Exciter filter: XF1073

Emitter filter: XF3084

Beam splitter: XF2010

Microscope cube set with the exciter filter, emitter filter and beam splitter: XF100-2

TROUBLESHOOTING

Observations	Suggestions
No colonies following transformation of XL10-Gold competent cells with recombinant pAAV plasmids	Perform a control transformation of the XL10-Gold cells using pUC18 control DNA to ensure that the cells have the expected transformation efficiency ($\geq 5 \times 10^9$ cfu/ μ g).
Low transfection efficiency for AAV-293 cells	Prepare highly purified DNA by CsCl gradient centrifugation.
	Verify that the pH of the 2 \times HBS buffer is precisely 7.1.
	The use of lipid-based transfection reagents is not recommended.
	Ensure that cells are healthy, evenly dispersed, and ~80% confluent when transfection is initiated.
Low titer of primary recombinant AAV stock	Check the transfection efficiency using the pAAV-LacZ or pAAV-hrGFP control. Low transfection efficiency may result in low virus titer.
	Ensure that the titer determination methods are accurate, using evenly-spread cells and counting faintly-staining cells as pAAV-LacZ ⁺ .
	Transgenes that encode products that are toxic to the host cells or that inhibit viral replication or packaging may produce low titer viral stocks.
	The use of the AAV-293 cells result in the highest viral titers. Do not replace with HEK 293 cells from a different source.
	Maintain the AAV-293 cells at <50% confluency prior to preparing viral particles.
	Insert size influences viral packaging efficiency. Use an insert between 1–3 kb when cloning into pAAV-MCS or pCMV-MCS. If the gene of interest is smaller than 1 kb, include “stuffer” DNA to produce an insert ≥ 1 kb.
Difficulty visualizing or counting X-gal-stained cells	Cells must be spread evenly in plates prior to cell staining assays. Avoid excessive swirling of plates, which tends to give cells a heterogeneous distribution.
	Use freshly prepared fixing and staining solutions.
	The use of etoposides is toxic to the cells and may cause them to round up or detach from the surface of the tissue culture dish when fixed and permeabilized using the reagents for detection of beta-galactosidase activity. Use care when generating the X-gal- stained cells.
	Increase the time of incubation in staining solution to up to 24 hours at 37°C under humidity.
Extensive HT1080 cell death during <i>Viral Titer Measurement of pAAV-LacZ AAV (Adenovirus-Free)</i> procedure	AAV permissive growth medium is toxic to cells. Ensure that 1) the concentration of camptothecin is correct, 2) cells are exposed to the Permissive Medium for ≤ 6 hours, 3) cells are $\geq 80\%$ confluent when treated, and 4) a wash step can be added after removing the chemical.
Low pAAV-IRES-hrGFP viral titer during <i>Viral Titer Measurement of pAAV-hrGFP AAV (Adenovirus-Free)</i> procedure	Expect a viral titer measurement approximately 10-fold lower than the titer of the recombinant pAAV-hrGFP-AAV; the hrGFP reporter is downstream from the IRES, and is therefore expressed at lower levels.
	The co-expressed gene of interest may be toxic to the AAV-HT1080 cells.
Cell lysis during <i>Viral Titer Measurement using Adenovirus Co-infection</i> procedure	Adjust the MOI for the helper adenovirus. Titrate the amount of adenovirus added to the co-infection suspensions over the MOI range of 10–100, holding the amount of pAAV-containing viral particles constant. Select the MOI that produces the highest titer without significant cell lysis.

(Table continues on the next page)

(Table continued from the previous page)

Observations	Suggestions
No expression of gene of interest in infected cells	Detection of expression of the gene of interest can be performed by transiently transfecting the double-stranded recombinant AAV plasmid into host cells and detecting expression of the protein of interest by immunodetection methods such as immunocytochemistry or Western blot analysis prior to generating viral particles and infecting cells. Transfection efficiency of the host cells can be determined using the pAAV-hrGFP vector and detecting expression of hrGFP. If expression of the gene of interest is not detected in the transient assay, it is unlikely that it will be detected later in infected cells.
	The single-stranded AAV genome must become double-stranded in order for gene expression to occur. Depending on the cell type, this can take from two to six weeks to occur. Increasing the MOI can accelerate gene expression.
	Heparan sulfate proteoglycan is the main attachment receptor for AAV. Co-receptors are fibroblast growth factor receptor 1 (FGFR 1) and $\alpha_v\beta_5$ integrin. Cells that lack these receptors may not transduce as well as cells that express these receptors at a high level.

PREPARATION OF MEDIA AND REAGENTS

<p>DMEM Growth Medium DMEM (containing 4.5 g/L glucose, 110 mg/L sodium pyruvate, and 2 mM L-glutamine) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 2 mM of L-glutamine</p>	<p>L-DMEM Growth Medium DMEM supplemented with 2% (v/v) heat-inactivated FBS and 2 mM L-glutamine</p>
<p>H-DMEM Growth Medium DMEM supplemented with 18% (v/v) heat-inactivated FBS and 2 mM L-glutamine</p>	<p>TE Buffer (1 ×) 10 mM Tris-HCl (pH 7.5) 1 mM EDTA Autoclave</p>
<p>Freezing Medium (100 ml) 50 ml DMEM (containing 4.5 g/L glucose, 110 mg/L sodium pyruvate and 2 mM L-glutamine) 40 ml heat-inactivated fetal bovine serum 10 ml dimethylsulfoxide (DMSO) Filter sterilize</p>	<p>Trypsin-EDTA Solution 0.53 mM tetrasodium ethylenediamine-tetraacetic acid (EDTA) 0.05% trypsin</p>
<p>2 × HBS 280 mM NaCl 1.5 mM Na₂HPO₄ 50 mM HEPES* Adjust the pH to 7.10 with NaOH *N-2-hydroxyethylpiperazine-N'-2ethanesulfonic acid</p>	<p>AAV Permissive Growth Medium DMEM Growth Medium (containing 10% (v/v) heat-inactivated FBS and 2mM L-glutamine) supplemented with 4.8 μM camptothecin. Prepare a stock of 1 mM camptothecin in PBS (from the original 10 mM camptothecin stock in DMSO), filter sterilize, then add 4.8 μl of the 1 mM camptothecin stock per ml of AAV Permissive Growth Medium. When handling camptothecin, follow the safety precautions suggested by the manufacturer or the MSDS.</p>
<p>PBS 137 mM NaCl 2.6 mM KCl 10 mM Na₂HPO₄ 1.8 mM KH₂PO₄ Adjust the pH to 7.4 with HCl</p>	

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ENDNOTES

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MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at <http://www.genomics.agilent.com>. MSDS documents are not included with product shipments.