VirusGEN® GMP AAV Transfection Kit

Protocol for MIR 6815-GMP

SDS available at mirusbio.com/6815

INTRODUCTION

Adeno-associated virus (AAV) is a nonenveloped, single-stranded DNA virus from the *Parvoviridae* family notable for its lack of pathogenicity, low immunogenicity and ability to infect both dividing and quiescent cells. Because AAV is replication-defective in the absence of adeno or helper proteins and is not implicated in any known human diseases, it is widely considered a safe gene delivery vehicle for *in vivo* and *in vitro* applications. Accordingly, recombinant AAV has become an invaluable tool for gene therapy and the creation of isogenic human disease models.

Efficient, high titer and large-scale viral vector manufacturing processes are necessary for production of AAV-based therapies. Additionally, raw or ancillary materials used for viral vector manufacturing must be carefully selected as part of a risk-based approach for the development of therapeutics. The *Trans*IT-VirusGEN® GMP Transfection Reagent addresses these needs by providing robust titers for AAV production, cGMP-compliant manufacturing and testing processes and expert support for researchers developing biotherapeutics. The VirusGEN® GMP AAV Transfection Kit further enhances the performance of *Trans*IT-VirusGEN® GMP Transfection Reagent in suspension HEK 293 cells through the addition of the proprietary VirusGEN® GMP AAV Complex Formation Solution and Enhancer. *Trans*IT-VirusGEN® GMP Transfection Reagent is assayed for formulation identity, appearance, sterility, bacterial endotoxin and mycoplasma. In addition to these tests, VirusGEN® GMP AAV Complex Formation Solution and Enhancer is assayed for pH and osmolality. Thus, *Trans*IT-VirusGEN® GMP AAV Transfection Kit is ideal for generating large-scale AAV preparations with the dependability and quality that are essential for streamlined manufacturing of gene and cell therapy products.

SPECIFICATIONS

Storage	Store *Trans*IT-VirusGEN® GMP Reagent at -10 to -30°C, tightly capped. Store VirusGEN® GMP AAV Complex Formation Solution and Enhancer at 2 to 10°C. ***Before each use***, warm to room temperature and mix gently.
Stability / Guarantee	Guaranteed as noted on the Certificate of Analysis when properly stored and handled.

Warm *Trans*IT-VirusGEN® GMP Reagent and VirusGEN® GMP AAV Complex Formation Solution and Enhancer to room temperature before each use. Mix gently.

MATERIALS

Materials Supplied

The VirusGEN® GMP AAV Transfection Kit (MIR 6815-GMP) is supplied in the following format.

Product No.	Component	Quantity
MIR 6845-GMP	*Trans*IT-VirusGEN® GMP Transfection Reagent	1 × 150 ml
MIR 6816-GMP	VirusGEN® GMP AAV Complex Formation Solution and Enhancer	5 × 1 L

For Materials Required but Not Supplied, See Protocol Sections:

- (I) AAV Generation in Suspension HEK 293 Cell Cultures
- (II) AAV Transduction/Titering Method Using a GFP Reporter Virus

For Research Use and Further Manufacturing; Not for Administration into Humans

BEFORE YOU START:

Important Tips for Optimal AAV Production

The suggestions below yield high efficiency plasmid DNA transfection using the VirusGEN® GMP AAV Transfection Kit.

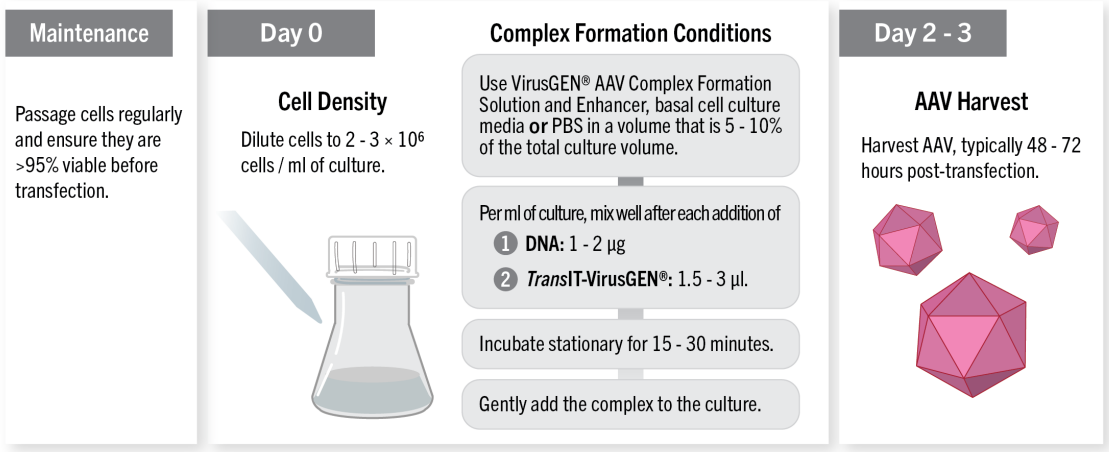
- **Cell culture conditions.** Use suspension HEK 293 cells with the VirusGEN® GMP AAV Transfection Kit. Before transfection, ensure cells are ≥ 95% viable by trypan blue exclusion and doubling every 24 hours. After transfection, there is no need to perform a medium change to remove the transfection complexes.
- **Cell density at transfection.** The recommended cell density is 2 - 3 × 10^6 cells/ml. Passage cells 18-24 hours before transfection to ensure that cells are actively dividing and reach the appropriate density at time of transfection.
- **AAV packaging and transfer plasmids.** The optimal ratio between plasmids will depend on the vector backbone and gene-of-interest. For each unique construct, empirically determine and use the optimal ratio for best results. Use plasmid manufacturer recommendations or previously established ratios as a starting point.
- **Ratio of *Trans*IT-VirusGEN® GMP to DNA.** Determine the optimal *Trans*IT-VirusGEN® GMP Reagent:DNA ratio for each cell type by varying the amount of reagent from 1.5-3 µl per 1-2 µg total DNA. Refer to **Table 1** for recommended starting conditions based on culture size.
- **Complex formation conditions.** Prepare *Trans*IT-VirusGEN® GMP Reagent:DNA complexes in VirusGEN® GMP AAV Complex Formation Solution and Enhancer, PBS or compatible basal cell culture media in a volume that is 5-10% of the total culture volume. For each unique vector construct, we recommend evaluating complex formation times between five minutes and one hour to identify an optimal time for maximal viral titer and quality. As a starting point, we recommend a complex formation time of 15-30 minutes. If forming complexes in a volume that is less than 5-10% of the total culture volume, complex formation time may need adjustments.

Premix packaging and transfer plasmids together prior to adding to the complex formation medium.

Do not use serum or antibiotics in the media during transfection complex formation.

Transfection complexes can be added directly to cells cultured in growth media +/- serum and up to 0.1-1X antibiotics.

VirusGEN® GMP AAV Transfection Kit Workflow:



SECTION I: AAV Generation in Suspension HEK 293 Cell Cultures

NOTE: Use of the VirusGEN® GMP AAV Complex Formation Solution and Enhancer is only recommended for AAV production in suspension HEK 293 cell lines. Contact Mirus Bio Technical Support for optimization in adherent cell culture platforms.

The following procedure describes plasmid DNA transfections for AAV generation in 125 ml Erlenmeyer shake flasks using 25 ml of complete growth medium. If using an alternate cell culture vessel, increase or decrease the amounts of VirusGEN® GMP AAV Complex Formation Solution and Enhancer, *Trans*IT-VirusGEN® GMP Reagent and total DNA based on the **volume of complete growth medium** to be used. To calculate the required reagent quantities based on the recommended starting conditions and total culture volume, refer to the calculation worksheet in **Table 1** (below).

Table 1. Calculation worksheet for scaling the VirusGEN® GMP AAV Transfection Kit

Starting conditions per milliliter of complete growth medium (AAV Generation)						
	Per 1 ml			Total culture volume		Reagent quantities
VirusGEN® GMP AAV Complex Formation Solution and Enhancer or PBS	0.1	ml	×	_____ml	=	_____ml
Total Plasmid DNA (1 µg/µl stock)	2	µl	×	_____ml	=	_____µl
*Trans*IT-VirusGEN® GMP Reagent	3	µl	×	_____ml	=	_____µl

NOTE: Total Plasmid DNA refers to the combined weight of AAV plasmids (in µg) per transfection.

Test PBS or compatible basal cell culture media in place of the VirusGEN® AAV Complex Formation Solution and Enhancer for each unique viral vector construct because, with some vectors, equivalently high titer can be obtained *without* the Enhancer component.

Materials Required but Not Supplied

- Suspension HEK 293 Cells (e.g. Viral Production Cells 2.0, Gibco Cat. No. A49784)
- Complete Culture Medium (e.g. Viral Production Medium (Gibco Cat. No. A4817901) or BalanCD HEK293 (Irvine Scientific Cat. No. 91165))
- Plasmid DNA (e.g. pAAV-hrGFP (Agilent Cat. No. 240074-51), pHelper (Agilent Cat. No. 240071-54), pALD-AAV5 (Aldevron Cat. No. 5058-10))
- Phosphate Buffered Saline (PBS) (e.g. Millipore Sigma Cat. No. D8537)
- Erlenmeyer shake flasks (e.g. Corning® Cat. No. 431143 or Thomson Cat. No. 931110)
- 10X Cell Lysis Buffer (500 mM Tris pH 8, 10% Tween® 20, 20 mM $MgCl_2$)
- 5 M Sodium Chloride (5 M NaCl)
- Benzonase® or equivalent (e.g. Sigma Cat. No. E1014 or Syd Labs Cat. No. BP4200)
- Reporter assay as required

Transient Plasmid Transfection Protocol per 25 ml HEK 293 Culture

A. Maintenance of cells

1. Passage suspension HEK 293 cells 18-24 hours prior to transfection to ensure that cells are actively dividing at the time of transfection and to obtain a density of 2 - 4 × 10^6 cells/ml the next day.
   NOTE: Perform cell counts and evaluate viability daily to ensure that cells are doubling every 24 hours and ≥ 95% viable by trypan blue exclusion. DO NOT proceed with transfection if cells are not doubling normally or are < 95% viable.
2. Incubate cells overnight at appropriate temperature and CO_2 levels (e.g. 37°C, 5-8% CO_2, shaking).

Passage cultured cells 18-24 hours before transfection to ensure that cells are actively dividing at the time of transfection.

B. Prepare *Trans*IT-VirusGEN® GMP:DNA complexes (immediately before transfection)

1. Immediately prior to transfection, seed cells at a density of 2 - 3 × 10^6 cells/ml into a transfection culture vessel (e.g. 25 ml per 125 ml Erlenmeyer shake flask).
2. Warm *Trans*IT-VirusGEN® GMP to room temperature and vortex gently before using.
3. Place 2.5 ml of VirusGEN® GMP AAV Complex Formation Solution and Enhancer in a sterile tube.
   NOTE: Mirus recommends comparing +/- Enhancer, i.e. forming complexes in 2.5 ml of PBS or compatible basal cell culture media. With some cell lines and vector constructs, equivalently high titer can be obtained *without* the Enhancer component.
4. In a separate sterile tube, combine AAV plasmids per manufacturer recommendations to a final concentration of 1 µg/µl. Mix thoroughly.
5. Transfer 50 µl of the DNA mixture prepared in Step B.4 to the tube containing VirusGEN® GMP AAV Complex Formation Solution and Enhancer. Mix completely.
6. Add 75 µl *Trans*IT-VirusGEN® GMP Reagent to the diluted DNA. Mix completely by inversion or vortexing. Do NOT agitate Reagent:DNA complexes again after initial mixing.
   NOTE: This is a 1.5:1 mixture of transfection reagent to total DNA (vol:wt), which can be further optimized for AAV production using *Trans*IT-VirusGEN® GMP Reagent.
7. Incubate the mixture at room temperature for 15-30 minutes stationary.
   NOTE: The ideal complex formation time may depend on the vector production platform but is typically between 5-60 minutes.

Do NOT allow the *Trans*IT-VirusGEN® GMP Reagent to incubate alone in complex formation solution > 5 min, i.e. if the reagent is pre-diluted, add DNA within 5 min for optimal complex formation.

Do NOT agitate Reagent:DNA complexes after the initial mixing. This will result in decreased titer.

C. Distribute the complexes to cells in complete growth medium

1. Add the *Trans*IT-VirusGEN® GMP:DNA complexes (prepared in Step B) to culture vessel, swirling gently to distribute.
2. Shake flasks on an orbital shaker (125 rpm when using a shaker with a 1.9 cm orbital throw) at appropriate temperature and CO_2 levels (e.g. 37°C, 5-8% CO_2).
3. Incubate cultures for 48-72 hours prior to AAV harvest.

There is no need to change culture medium after transfection, unless required by your cell type or culture conditions.

D. Harvest and storage of AAV

1. Following the 48-72 hour incubation, transfer the total volume of cell suspension (i.e. 27.5 ml) to a sterile conical tube or appropriate vessel.
2. Add 0.1X volume of 10X Cell Lysis Buffer (i.e. 2.75 ml) and 100 U/ml Benzonase® (i.e. 2,750 U). Mix completely and incubate at 37°C for 1.5 hours with shaking.
3. Add 0.1X volume of 5 M NaCl (i.e. 2.75 ml) and mix completely. Incubate at 37°C for 30 minutes with shaking.
4. Centrifuge the mixture at 4,100 × g for 10 minutes to remove cell debris. Carefully transfer the AAV containing supernatant to a new sterile tube.
5. Store AAV stocks at -80°C.

Benzonase® is a non-specific endonuclease used to liberate virus particles from residual nucleic acids in the cell lysates and increase AAV titers.

SECTION II: AAV Transduction/Titering Method Using a GFP Reporter Virus

The following procedure describes transduction of HT-1080 cells grown in a 24-well format with a GFP reporter AAV2 and is meant to determine functional AAV2 titers. The number of wells needed for this assay will depend on the number of AAV2 stocks titered and the number of dilutions required for testing per stock (see Step B.3). Testing several dilutions is recommended to accurately determine the functional AAV2 titer. This protocol can be adapted to transduce cells that are permissive to different AAV serotypes.

Materials Required, but Not Supplied

- HT-1080 cells (ATCC Cat. No. CCL-121)
- Dulbecco's Modified Eagle Medium (DMEM) (Corning Cat. No. 10-013-CV)
- Complete HT-1080 cell culture medium (e.g. DMEM + 10% FBS)
- DMEM + 2% FBS for AAV dilutions
- AAV stock(s) expressing GFP reporter
- 24-well tissue culture plate(s)
- 1X PBS and trypsin
- Flow cytometer equipped with a GFP compatible laser

A. Plate cells

1. Approximately 4-6 hours before transduction, plate HT-1080 cells in 0.5 ml complete growth medium per well in a 24-well plate. A starting cell density of 1×10^5 cells/ml is recommended. Cells should be adhered to the plate and 40-50% confluent at the time of transduction.
2. Record the cell count, which is critical to determine an accurate functional titer.

B. Transduce with GFP-encoding recombinant AAV

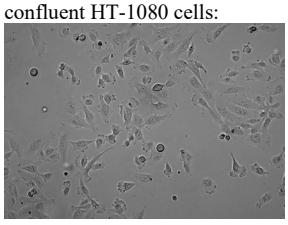
1. Thaw AAV stock(s) in 37°C water bath. Remove promptly after virus has thawed to prevent virus inactivation. Gently mix virus stock.
2. Make 1:250 and 1:1000 dilutions of the AAV stock(s) in DMEM + 2% FBS.
   NOTE: Each test well will receive 50 µl of the appropriate dilution. Lower or higher dilutions may be required depending on the serotype and AAV production conditions.
3. Add 50 µl of the appropriate AAV dilution to wells containing cells.
4. Incubate the assay wells at 37°C in 5% CO_2 for 48 hours post-transduction.
5. NOTE: To obtain an accurate titer, it is desirable to have less than 20% GFP positive cells at 48 hours post-transduction. This minimizes counting cells with multiple integration events, which would result in an underestimation of titer.

C. Cell harvest and analysis

1. Gently wash cells with 200 µl 1X PBS. Following the removal of PBS, immediately add 100 µl of trypsin to each well.
2. Incubate the plate at 37°C and closely monitor cell rounding and detachment.
3. After cells have detached, add 400 µl of complete growth media (e.g. DMEM + 10% FBS) to each well to inactivate the trypsin and resuspend the cells. NOTE: The cells should be at ~ 1×10^6 cells/ml. The cells can be further diluted in growth media if desired.
4. Transfer 250 µl of cell suspension from each well to separate wells in a non-treated 96-well plate (or similar culture vessel) that is compatible with your flow cytometer.
   NOTE: The optimal volume added for dilution may vary depending on the flow cytometer.

Cells can also be plated 18-24 hours before transduction, but at a lower density (7.5×10^4 cells/ml) to ensure 40-50% confluency at the time of transduction. If plated the day before, the cells should be trypsinized and counted again at the time of transduction because cells will likely have divided and increased in number.

Representative image of ~50% confluent HT-1080 cells:



5. Analyze for GFP expression by flow cytometry.
6. Calculate the functional titer of the AAV stock using the following equation:

$$\text{Titer (HT-1080 Transducing units/ml)} = \left[\frac{\text{Number of target cells (Count at time of transduction)} \times [\%\ \text{GFP positive cells}/100]}{\text{(Volume of AAV Stock in ml)}} \right.$$

NOTE: To determine the functional titer produced per milliliter of total culture, multiply the AAV stock titers determined above by the dilution factor at harvest (e.g. for AAV produced in suspension HEK 293 cultures and harvested as described in Section I.D, multiply AAV stock titers determined above by 1.2).

TROUBLESHOOTING GUIDE

POOR DNA TRANSFECTION EFFICIENCY	
Problem	**Solution**
Incorrect vector sequence	If you do not observe expression of your target insert, verify the sequence of your plasmid DNA.
Suboptimal *Trans*IT® Reagent:DNA complex formation conditions	Determine the best *Trans*IT-VirusGEN® GMP Reagent:DNA ratio for each viral vector construct. Titrate the *Trans*IT-VirusGEN® GMP Reagent volume from 1-3 µl per 1 µg DNA. Refer to "Before You Start" on Page 2 for recommended starting conditions.
	Test PBS or compatible basal cell culture media in place of the VirusGEN® AAV Complex Formation Solution and Enhancer for each unique viral vector construct because, in some cases, equivalently high titer can be obtained *without* the Enhancer component.
Suboptimal DNA concentration	Determine DNA concentration accurately. Use plasmid DNA with an $A_{260/280}$ of 1.8-2.0.
	The optimal DNA concentration generally ranges between 0.5-2 µg per 1 ml of culture. Start with 2 µg DNA per 1 ml of culture. Consider testing different amounts of DNA while scaling the amount of *Trans*IT-VirusGEN® GMP accordingly.
Low-quality plasmid DNA	Use highly purified, sterile, endotoxin- and contaminant-free DNA for transfection.
	Remove endotoxin from your DNA preparation. Use cesium chloride gradient or anion exchange purified DNA which contains levels of endotoxin that do not harm most cells.
Cells not actively dividing at the time of transfection	Divide the culture at least 18-24 hours before transfection to ensure that the cells are actively dividing and reach optimal cell density at time of transfection. DO NOT proceed with transfection if cells are not doubling normally or are < 95% viable by trypan blue exclusion.
Time of AAV harvest not optimal	Determine the optimal time to harvest AAV post-transfection. Though typically 48-72 hours post-transfection, the best time to harvest will depend on the vector construct and production platform.
*Trans*IT-VirusGEN® GMP was not mixed properly	Warm *Trans*IT-VirusGEN® Reagent to room temperature and mix gently before each use.
	If *Trans*IT-VirusGEN® GMP Reagent is pre-diluted in complex formation solution, DNA should be added within 5 min. Incubating the *Trans*IT-VirusGEN® GMP Reagent in complex formation solution alone for an extended time results in reduced production of functional virus.
Disruption of transfection complex formation	After initial mixing of DNA and *Trans*IT-VirusGEN® GMP Reagent, do not agitate Reagent:DNA complexes again, e.g. do not vortex or mix immediately before adding to cultures.
Excessive complex formation time	We recommend a complex formation time of 15-30 minutes, though viral titer and quality may be further optimized by evaluating complex formation times between 5-60 minutes for each unique vector construct.
Precipitate formation or turbid appearance during transfection complex formation	During complex formation, scale all reagents according to the table in the protocol, including serum-free media, *Trans*IT-VirusGEN® GMP Reagent and plasmid DNA.
	Precipitation may be observed when excess DNA is used during complex formation. This may negatively impact transfection efficiency. To avoid precipitation when using high concentrations of DNA, increase the volume of serum-free medium used during complex formation.
	Large-volume transfection complexes may appear turbid – typically, this phenomenon does *not* negatively impact transfection as long as complexes are well mixed.
Proper experimental controls were not included	To assess delivery efficiency of plasmid DNA, use Mirus *Label* IT® Tracker™ Intracellular Nucleic Acid Localization Kit to label the target plasmid or use Mirus prelabeled *Label* IT® Plasmid Delivery Controls (please refer to Related Products on Page 9).
	To verify efficient transfection, use *Trans*IT-VirusGEN® GMP Reagent to deliver a positive control such as a luciferase, beta-galactosidase or green fluorescent protein encoding plasmid.

TROUBLESHOOTING GUIDE continued

HIGH CELLULAR TOXICITY	
Problem	**Solution**
Cell density not optimal at time of transfection	High toxicity and cell death may be observed if cells are not dense at the time of transfection. For high virus titers using *Trans*IT-VirusGEN® GMP Reagent, ensure that cell cultures are approximately 2 - 3 × 10^6 cells/ml (for suspension cell transfections) at the time of transfection.
Cell morphology has changed	When generating AAV with the VirusGEN® GMP AAV Complex Formation Solution and Enhancer, cell growth and viable cell density may decrease 48-72 hours post-transfection. This is normal and does not adversely affect virus titers.
	Mycoplasma contamination can alter cell morphology and affect transfection efficiency. Check your cells for mycoplasma contamination. Use a fresh frozen stock of cells or use appropriate antibiotics to eliminate mycoplasma.
	A high or low cell passage number can make cells more sensitive and refractory to transfection. Maintain adherent or suspension HEK 293 cells below passage 30 for optimal recombinant virus production.
Transfection complexes not evenly distributed after complex addition to cells	Add transfection complexes while swirling the flask. If this is not possible, gently mix the culture vessel to ensure even distribution of the transfection complexes. However, avoid vigorous agitation that could disturb formed transfection complexes, e.g. vortexing after the initial mixing of the DNA and transfection reagent.

RELATED PRODUCTS

- VirusGEN® AAV Transfection Kit
- VirusGEN® GMP LV Transfection Kit
- VirusGEN® LV Transfection Kit
- *Trans*IT-VirusGEN® GMP Transfection Reagent
- *Trans*IT-VirusGEN® Transfection Reagent
- *Transduce*IT™ Reagent
- *Label* IT® Plasmid Delivery Controls
- *Label* IT® Tracker™ Intracellular Nucleic Acid Localization Kits
- MiraCLEAN® Endotoxin Removal Kits
- Ingenio® Electroporation Solution and Kits

For details on the above-mentioned products, visit www.mirusbio.com

Reagent Agent®

Reagent Agent® is an online tool designed to help determine the best solution for nucleic acid delivery based on in-house data, customer feedback and citations.

Learn more at:
www.mirusbio.com/ra

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