VirusGEN® AAV Transfection Kit

Protocol for MIR 6750

Quick Reference Protocol, SDS and Certificate of Analysis available at mirusbio.com/6750

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.

The *Trans*IT-VirusGEN® Transfection Reagent enables the generation of high titer AAV in HEK 293 cell types. The VirusGEN® AAV Transfection Kit further enhances the performance of *Trans*IT-VirusGEN® Transfection Reagent in suspension HEK 293 cells through the addition of the proprietary VirusGEN® AAV Complex Formation Solution and Enhancer. The *Trans*IT-VirusGEN® AAV Transfection Kit is ideal for generating high titer AAV preparations to accelerate research and development.

SPECIFICATIONS

Storage	Store *Trans*IT-VirusGEN® Reagent at -10 to -30°C, tightly capped. Store VirusGEN® AAV Complex Formation Solution and Enhancer at 2 to 10°C. ***Before each use***, warm to room temperature and mix gently.
Stability / Guarantee	6 months from the date of purchase, when properly stored and handled.

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

MATERIALS

Materials Supplied

The VirusGEN® AAV Transfection Kit (MIR 6750) is supplied in the following format.

Product No.	Component	Quantity
MIR 6702A	*Trans*IT-VirusGEN® Transfection Reagent	2 × 1.5 ml
MIR 6751A	VirusGEN® AAV Complex Formation Solution and Enhancer	1 × 100 ml

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 Only

BEFORE YOU START:

Important Tips for Optimal AAV Production

Mirus recommends using suspension Expi293F™ Cells (Gibco Cat. No. A14527) for high titer AAV production. The suggestions below yield high efficiency plasmid DNA transfection using the VirusGEN® AAV Transfection Kit.

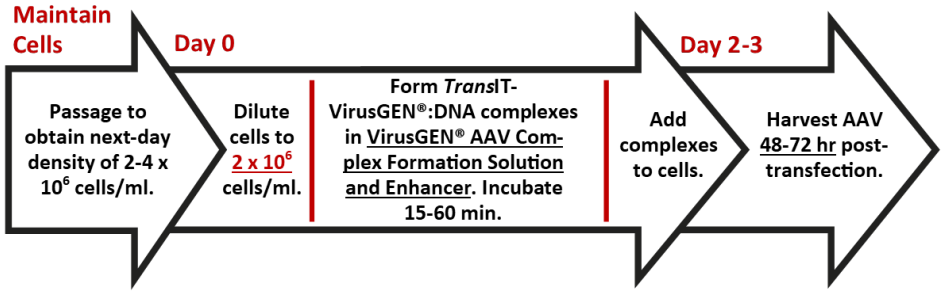
- **Cell density at transfection.** The recommended cell density for suspension Expi293F™ cells is 2×10^6 cells/ml. Passage cells 18-24 hours before transfection to ensure that cells are actively dividing and reach the appropriate density at time of transfection.
- **DNA purity.** Use highly purified, sterile, endotoxin-free and contaminant-free DNA for transfection. Plasmid DNA preparations that have an $A_{260/280}$ absorbance ratio of 1.8-2.0 are desirable. DNA prepared using miniprep kits is not recommended as it might contain high levels of endotoxin. We recommend using MiraCLEAN® Endotoxin Removal Kit (MIR 5900) to remove endotoxin from your DNA preparation.
- **AAV packaging and transfer plasmids.** The VirusGEN® AAV Transfection Kit was optimized using a 1:1:1 weight ratio of pAAV-hrGFP, pAAV-RC and pHelper (AAV Helper-Free System, Agilent Technologies). The optimal ratio may vary for other packaging systems.
- **Ratio of *Trans*IT-VirusGEN® to DNA.** Determine the optimal *Trans*IT-VirusGEN® 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® Reagent:DNA complexes in VirusGEN® AAV Complex Formation Solution and Enhancer. We recommend forming complexes in a volume that is 10% of the total culture volume. If forming complexes in a smaller or larger volume, complex formation time may need to be shortened or extended. We recommend an initial complex formation time of 30 minutes (15-60 minutes yields similar results).
- **Cell culture conditions.** Culture cells in the appropriate complete growth medium, (e.g. Expi293™ Expression Medium). 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.
- **Presence of antibiotics.** Antibiotics inhibit transfection complex formation and should be excluded from the complex formation step. Transfection complexes can be added directly to cells growing in complete culture medium containing serum and low levels of antibiotics (0.1-1X final concentration of penicillin/streptomycin mixture).
- **Media change post-transfection.** A media change is not required and could be detrimental to virus titers; therefore, we do not recommend a media change post-transfection.
- **Post-transfection incubation time.** The optimal incubation time for harvesting high titer AAV generated with this kit is generally 48-72 hours post-transfection.

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® AAV Transfection Kit Workflow:



SECTION I: AAV Generation in Suspension HEK 293 Cell Cultures

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® AAV Complex Formation Solution and Enhancer, *Trans*IT-VirusGEN® 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).

NOTE: Use of the VirusGEN® AAV Complex Formation Solution and Enhancer is only recommended for AAV production in suspension HEK 293 cell lines.

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

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

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

Complexes can also be formed in PBS or serum-free basal medium instead of the VirusGEN® AAV Complex Formation Solution and Enhancer. However, doing so may result in decreased AAV titer.

Materials Required but Not Supplied

- Suspension HEK 293 Cells (e.g. Expi293F™ Cells, Gibco Cat. No. A14527)
- Complete Culture Medium (e.g. Expi293™ Expression Medium, Gibco Cat. No. A1435101)
- Plasmid DNA (e.g. Agilent AAV2 pDNA: pAAV-hrGFP (Cat. No. 240074-51), pAAV-RC (Cat. No. 240071-53), pHelper (Cat. No. 240071-54))
- 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® (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).

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

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

1. Immediately prior to transfection, seed cells at a density of 2 × 10^6 cells/ml into a transfection culture vessel (e.g. 25 ml per 125 ml Erlenmeyer shake flask).
2. Warm *Trans*IT-VirusGEN® to room temperature and vortex gently before using.
3. Place 2.5 ml of VirusGEN® AAV Complex Formation Solution and Enhancer in a sterile tube.
   NOTE: When evaluating new AAV serotypes or plasmid constructs, Mirus recommends comparing +/- Enhancer, i.e. forming complexes in 2.5 ml of PBS.
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® AAV Complex Formation Solution and Enhancer. Mix completely.
6. Add 75 µl *Trans*IT-VirusGEN® Reagent to the diluted DNA. Mix completely. Mix completely by inversion or vortexing. Do NOT agitate Reagent:DNA complexes again after this 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 at room temperature for 15-60 minutes without additional agitation to allow transfection complexes to form.

Do NOT allow the *Trans*IT-VirusGEN® 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®: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.
   NOTE: See 'Freeze/thaw Procedure' below for an alternative method to harvest AAV.
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.

Harvest of AAV with Freeze/thaw Procedure (Alternative)

1. Prepare a dry ice/ethanol bath.
2. Centrifuge cell suspension at 1,750 × g for 10 minutes.
3. Remove the supernatant and process as needed.
4. Add 5.5 ml of Freeze/Thaw Lysis Buffer (50 mM Tris pH 8.5, 150 mM NaCl, 2 mM $MgCl_2$) to the cell pellet. Mix thoroughly until cell clumps are no longer visible.
   NOTE: The required volume of Freeze/Thaw Lysis Buffer is calculated by multiplying the transfected cell culture volume by 0.2 ml.
5. To ensure sufficient lysis of the cells, freeze cells completely in the dry ice/ethanol bath. Visually inspect to verify that cells are frozen solid, then thaw in a 37°C water bath. Repeat freeze/thaw procedure a total of three times.
6. Add 50 U/ml Benzonase® (i.e. 275 U) to the cell lysate and mix gently but thoroughly. Incubate at room temperature for 30 minutes.
7. Centrifuge the cell lysate at 10,000 × g for 10 minutes to remove cell debris. Carefully transfer the AAV containing supernatant to a new sterile tube.
8. Store AAV stocks at -80°C.

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.2). 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.

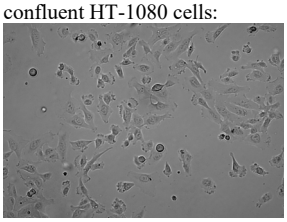
   NOTE: To obtain an accurate titer, it is desirable to have less than 20% GFP positive cells at 48 hours post-transduction. This minimizes counting cells with multiple integration events, which would result in an underestimation of titer.

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 the transfer vector plasmid DNA.
Suboptimal *Trans*IT® Reagent:DNA ratio	Determine the best *Trans*IT-VirusGEN® Reagent:DNA ratio for each cell type. Titrate the *Trans*IT-VirusGEN® Reagent volume from 1-3 µl per 1 µg DNA. Refer to "Before You Start" on Page 2 for recommended starting conditions.
Suboptimal DNA concentration	Determine the DNA concentration accurately. Use plasmid DNA preps that have an $A_{260/280}$ absorbance ratio 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® accordingly.
Low-quality plasmid DNA	Use highly purified, sterile, endotoxin- and contaminant-free DNA for transfection. We recommend using Mirus MiraCLEAN® Endotoxin Removal Kit (MIR 5900) for removal of endotoxin from your DNA preparation. Alternatively, use cesium chloride gradient or anion exchange purified DNA which contains levels of endotoxin that do not harm most cells. **Do not** use DNA prepared using miniprep kits as it might contain high levels of endotoxin.
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.
Transfection incubation time not optimal	Determine the optimal transfection incubation time for each cell type and experiment. Test a range of incubation times (e.g. 48-72 hours). The best post-transfection incubation time for AAV is typically 48-72 hours.
TransIT-VirusGEN® was not mixed properly	Warm *Trans*IT-VirusGEN® Reagent to room temperature and vortex gently before each use. If *Trans*IT-VirusGEN® Reagent is pre-diluted in complex formation solution, DNA should be added within 5 min. Incubating the *Trans*IT-VirusGEN® 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® Reagent, do not agitate Reagent:DNA complexes again, e.g. do not vortex or invert immediately before adding to cultures.
Precipitate formation during transfection complex formation	During complex formation, scale all reagents according to the scaling tables provided in each section of the protocol, including: serum-free media, *Trans*IT-VirusGEN® 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 during complex formation by two-fold.
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 10). To verify efficient transfection, use *Trans*IT-VirusGEN® Reagent to deliver a positive control such as a luciferase, beta-galactosidase or green fluorescent protein (GFP) 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® Reagent, ensure that cell cultures are approximately 2 × 10^6 cells/ml (for suspension cell transfections) at the time of transfection.
Cell morphology has changed	When generating AAV with the VirusGEN® AAV Complex Formation Solution and Enhancer, cell growth may decrease. 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.

RELATED PRODUCTS

- VirusGEN® GMP AAV Transfection Kit
- VirusGEN® SELECT AAV Transfection Kit
- VirusGEN® GMP LV Transfection Kit
- VirusGEN® SELECT LV Transfection Kit
- VirusGEN® LV Transfection Kit
- *Trans*IT-VirusGEN® GMP Transfection Reagent
- *Trans*IT-VirusGEN® SELECT 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®

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