VirusGEN® and *Rev*IT™ GMP AAV Transfection Kit

Protocol for MIR 8210-GMP

SDS available at mirusbio.com/literature

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 adenovirus 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 the 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 addition of *Rev*IT™ GMP AAV Enhancer further heightens the performance of *Trans*IT-VirusGEN® GMP Transfection Reagent in suspension HEK 293 cells.

*Trans*IT-VirusGEN® GMP Transfection Reagent is assayed for formulation identity, density, appearance, sterility, bacterial endotoxin and mycoplasma. In addition to these tests, *Rev*IT™ GMP AAV Enhancer is assayed for purity. Thus, the VirusGEN® and *Rev*IT™ 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.

Warm all reagents to room temperature and mix gently before each use.

Ensure room temperature of >20°C when working with, and handling, *Rev*IT™ GMP AAV Enhancer post-thaw.

NOTE: *Rev*IT™ GMP AAV Enhancer remains frozen at temperatures < 19°C.

SPECIFICATIONS

Storage	Store *Trans*IT-VirusGEN® GMP Transfection Reagent at -10 to -30°C, tightly capped. ***Before each use***, warm to room temperature and vortex gently. Return to proper storage conditions after each use.
	Store *Rev*IT™ GMP AAV Enhancer at -10 to -30°C, tightly capped. ***Before use***, thaw completely at >20°C and vortex gently. Return to proper storage conditions after use.
Stability / Guarantee	Guaranteed as noted on the Certificate of Analysis when properly stored and handled.

MATERIALS

Materials Supplied

The VirusGEN® and *Rev*IT™ GMP AAV Transfection Kit is supplied in the following format:

Product No.	Component	Quantity
MIR 6845-GMP	*Trans*IT-VirusGEN® GMP Transfection Reagent	4 × 150 ml
MIR 8200-GMP	*Rev*IT™ GMP AAV Enhancer	1 × 200 ml

For Materials Required, but Not Supplied, See Page 3.

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® and *Rev*IT™ GMP AAV Transfection Kit.

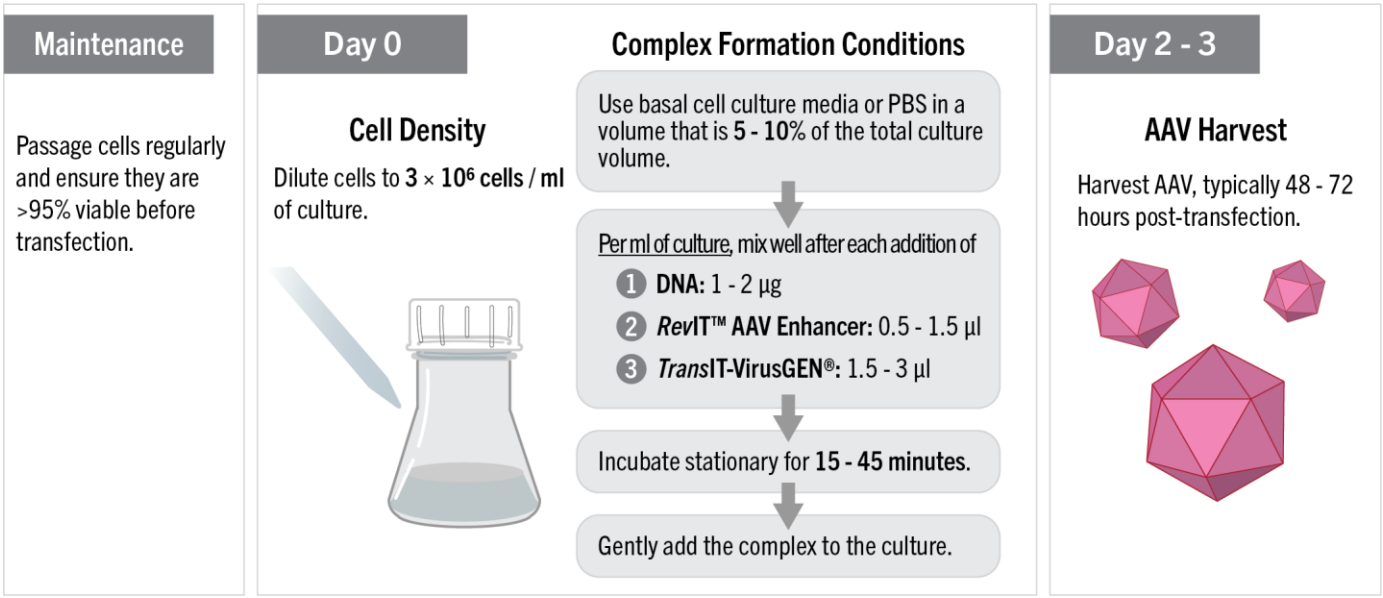
- **Cell culture conditions.** Use suspension HEK 293 cells with the VirusGEN® and *Rev*IT™ GMP AAV Transfection Kit. Before transfection ensure cells are ≥ 95% viable by trypan blue exclusion (or similar method) and doubling every 24 hours. After transfection, there is no need to perform a medium change to remove the transfection complexes.
- **Cell density at transfection.** The recommended cell density is 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.
- ***Rev*IT™ GMP AAV Enhancer.** Titrate *Rev*IT™ GMP AAV Enhancer from 0.5 to 1.5 µl per 1 ml of culture to determine the optimal amount for production of your specific viral vector. *Rev*IT™ GMP AAV Enhancer, MIR 8200-GMP, may take ~36 to 48 hours to thaw completely at room temperature (20°C) prior to use.
- **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 of total DNA. Refer to **Table 1** for recommended starting conditions based on culture size.
- **Complex formation conditions.** Prepare *Trans*IT-VirusGEN® GMP Reagent:*Rev*IT™ GMP AAV Enhancer:DNA complexes in PBS or compatible basal cell culture media in a volume that is 5-10% of the total culture volume. We recommend a complex formation time of 15-45 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® and *Rev*IT™ GMP AAV Transfection Kit Workflow:



AAV GENERATION IN SUSPENSION HEK 293 CELL CULTURES

NOTE: Use of the VirusGEN® and *Rev*IT™ GMP AAV Transfection Kit 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 30 ml of complete growth medium. If using an alternate cell culture vessel, increase or decrease the amounts of serum-free complex formation medium, *Trans*IT-VirusGEN® GMP Reagent, *Rev*IT™ GMP AAV Enhancer 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. Scaling worksheet for *Trans*IT-VirusGEN® GMP with *Rev*IT™ GMP AAV Enhancer

Starting conditions per milliliter of complete growth medium					
	Per 1 ml		Total culture volume		Reagent quantities
PBS or Basal Medium	0.1 ml	×	_____ ml	=	_____ ml
Total Plasmid DNA (1 µg/µl stock)	2 µl	×	_____ ml	=	_____ µl
*Rev*IT™ GMP AAV Enhancer	1 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.

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), AAV8 Rep-Cap Plasmid (GeneMedi Cat. No. P-RC09))
- 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)

www.mirusbio.com | techsupport@mirusbio.com | U. S. Toll-Free: 844.647.8724 | Direct: 608.441.2852

Transient Transfection Protocol per 30 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 3 - 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 under appropriate conditions (e.g. 37°C, 5-8% CO_2, shaking).

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

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

1. Warm *Trans*IT-VirusGEN® GMP Reagent and *Rev*IT™ GMP AAV Enhancer to room temperature and vortex gently before using. When thawing at room temperature, allow ~36 to 48 hours and ensure ambient air temperature is > 19°C.
2. Immediately prior to transfection, seed cells at a density of 3 × 10^6 cells/ml into a transfection culture vessel (e.g. 30 ml per 125 ml Erlenmeyer shake flask).
3. Place 3 ml of PBS in a sterile tube.
4. In a separate sterile tube, combine AAV plasmids per manufacturer recommendations to a final concentration of 1 µg/µl. Mix thoroughly.
5. Transfer 60 µl of the DNA mixture prepared in Step B.4 to the tube containing PBS. Mix completely.
6. Add 30 µl of *Rev*IT™ GMP AAV Enhancer to the diluted DNA and PBS. Mix completely.
7. Add 90 µl of *Trans*IT-VirusGEN® GMP Reagent to the diluted DNA:*Rev*IT™ mixture. Mix completely by inversion or vortexing. Do NOT agitate *Trans*IT-VirusGEN® GMP:*Rev*IT™ GMP:DNA complexes again after this initial mixing.
8. Incubate at room temperature for 15-45 minutes without additional agitation to allow transfection complexes to form.

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

Do NOT agitate *Trans*IT-VirusGEN® GMP:*Rev*IT™ GMP: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:*Rev*IT™ 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. 33 ml) to a sterile conical tube or appropriate vessel.
2. Add 0.1X volume of 10X Cell Lysis Buffer (i.e. 3.3 ml) and 100 U/ml Benzonase® (i.e. 3,300 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. 3.3 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.

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:*Rev*IT™:DNA ratio	Determine the best *Trans*IT-VirusGEN® GMP Reagent:*Rev*IT™ GMP AAV Enhancer:DNA ratio for each cell type. Titrate the *Trans*IT-VirusGEN® GMP Reagent volume from 1-3 µl per 1 µg of DNA. Titrate the *Rev*IT™ GMP AAV Enhancer volume from 0.5-1.5 µl per 1 ml of culture. Refer to "Before You Start" on Page 2 for recommended starting conditions.
Suboptimal DNA concentration	Determine the DNA concentration accurately. Use plasmid DNA preps 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.
	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® GMP Reagent to room temperature and vortex gently before each use.
	If *Trans*IT-VirusGEN® GMP Reagent is pre-diluted in complex formation solution, DNA should be added within 5 minutes. 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, *Rev*IT™ GMP AAV Enhancer and *Trans*IT-VirusGEN® GMP Reagent, do not agitate the Reagent:Enhancer:DNA complexes again, e.g. do not vortex or invert before adding to cultures.
Precipitate formation 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, *Rev*IT™ GMP AAV Enhancer 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.
	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 7).
	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 (GFP) encoding plasmid.

www.mirusbio.com | techsupport@mirusbio.com | U. S. Toll-Free: 844.647.8724 | Direct: 608.441.2852

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 3×10^6 cells/ml (for suspension cell transfections) at the time of transfection.
Cell morphology has changed	When generating AAV with *Rev*IT™ GMP AAV 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. However, avoid vigorous agitation that could disturb formed transfection complexes, e.g. vortexing after the initial mixing of the DNA, enhancer and transfection reagent.

www.mirusbio.com | techsupport@mirusbio.com | U. S. Toll-Free: 844.647.8724 | Direct: 608.441.2852

RELATED PRODUCTS

- *Trans*IT-VirusGEN® GMP Transfection Reagent
- *Trans*IT-VirusGEN® Transfection Reagent
- *Rev*IT™ GMP AAV Enhancer
- *Rev*IT™ AAV Enhancer
- VirusGEN® GMP AAV Transfection Kit
- VirusGEN® AAV Transfection Kit
- *Label* IT® Plasmid Delivery Controls
- *Label* IT® Tracker™ Intracellular Nucleic Acid Localization Kits
- MiraCLEAN® Endotoxin Removal Kits

For details on the above-mentioned products, visit 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: mirusbio.com/ra

Contact Mirus Bio for additional information.

Mirus Bio LLC
5602 Research Park Blvd, Ste 210
Madison, WI 53719
Toll-free: 888.530.0801
Direct: 608.441.2852
Fax: 608.441.2849