

AAViator™ GMP Transfection System

MIR 73720-GMP

SDS available at mirusbio.com/literature

INTRODUCTION

Adeno-associated virus (AAV) is a non-enveloped, 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. AAV is not implicated in any known human diseases, it is widely considered a safe gene delivery vehicle for *in vitro* and *in vivo* applications. Accordingly, recombinant AAV has become an invaluable tool for gene therapy and the creation of isogenic human disease models.

The AAViator™ GMP Transfection System enables the generation of high titer AAV in suspension HEK 293 cell types while often improving the full/empty capsid ratio and reducing the amount of plasmid DNA required per transfection.

The AAViator™ GMP Transfection System includes:

- TransIT® AAViator GMP Transfection Reagent proven for high titer AAV production in suspension HEK 293 derived cells.
- RevIT™ GMP AAV Enhancer designed to increase AAV titer 2-4X for any serotype, reagent, HEK 293 cell line or media.

TransIT® AAViator GMP Transfection Reagent is intended to be used with RevIT™ GMP AAV Enhancer as yields are highest when used together.

SPECIFICATIONS

Storage	Store TransIT® AAViator GMP Transfection Reagent and RevIT™ GMP AAV Enhancer at -10 to -30°C, tightly capped.
Stability / Guarantee	Guaranteed as noted on the Certificate of Analysis when properly stored and handled.



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

NOTE: RevIT™ GMP AAV Enhancer remains frozen at temperatures < 19°C as it is a DMSO solution.

MATERIALS

Product No.	Component	Quantity
MIR 73780-GMP	TransIT® AAViator GMP Transfection Reagent	1 x 450 mL
MIR 8200-GMP	RevIT™ GMP AAV Enhancer	1 x 200 mL

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

BEFORE YOU START:

THE FOLLOWING CONTAINS SUGGESTIONS FOR GUIDANCE PURPOSES ONLY AND SHOULD BE EVALUATED BY EACH CUSTOMER PRIOR TO ESTABLISHING IN A PROCESS IN A GMP SETTING. THIS PROTOCOL HAS NOT BEEN QUALIFIED NOR VALIDATED.

Important Tips for Optimal AAV Production

The suggestions below yield high-efficiency plasmid DNA transfection using the AAViator™ GMP Transfection System.

- **Cell culture conditions.** Culture suspension HEK 293 cells in appropriate complete growth medium, such as Cellvento 4HEK® (+ 6mM L-glutamine). Ensure cells are ≥ 95% viable 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 but the optimal density should be empirically determined for each cell type.
 - Utilize a seeding and cell passage strategy that ensures cells are actively dividing and at the appropriate density before transfection. Follow cell manufacturer's recommendations for the maximum and minimum cell density.
- **AAV packaging and transfer plasmids.** The optimal ratio between plasmids will depend on the vector backbone and gene-of-interest.
 - For each unique construct, use plasmid manufacturer recommendations or previously established ratios. A 1:1:1 mass ratio can also be used as a starting point.
 - Mirus recommends resuspending plasmids in water.
- **Ratio of TransitIT® AAViator GMP to DNA.** The optimal ratio between TransitIT® AAViator GMP Reagent and DNA is typically 1:1 to 1.5:1, therefore, the recommendation is to start at 1.25:1 (vol:wt).
 - To determine the optimal ratio for your unique cell culture system, evaluate AAV production using the following ranges of reagent and DNA per mL of culture:
 - TransitIT® AAViator GMP:** 1 to 2.3 µL
 - Total DNA:** 1 to 1.5 µg
- **RevIT™ GMP AAV Enhancer thaw time.** It is recommended to test and establish thaw times and appropriate freeze/thaw cycles within any process. The following was established from testing of RUO grade material.
 - As a guideline, RevIT™ AAV enhancer takes ~48 hours to thaw at room temperature, or ~5.5 hours in a 37°C incubator.
 - As a guideline, RevIT™ AAV Enhancer is known to maintain function through at least five freeze-thaw cycles (thawed in a 37°C incubator). Return to proper storage conditions after each use.
- **RevIT™ GMP AAV Enhancer.** Titrate RevIT™ GMP AAV Enhancer from 0.5 to 1.5 µL per 1 mL of culture. Use 1 µL per mL as a starting point.
- **Complex formation conditions.** Prepare transfection complexes in compatible basal cell culture media in a volume that is 5% of the total culture volume.
 - The optimal complex formation time may depend on the complex formation solution utilized.



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.

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Materials Required, But Not Supplied

- Suspension HEK 293 Cells (e.g. Viral Production Cells 2.0, Gibco A49784)
- Complete Culture Medium (e.g. Viral Production Medium (VPM), Thermo Fisher A4817901 or Cellvento 4HEK®, MilliporeSigma 125193). Supplement with 4 mM (VPM) or 6 mM (Cellvento) L-glutamine (MilliporeSigma 59202C) or GlutaMAX™ (Gibco 35050061).
- Plasmid DNA (e.g. pALD-ITR-WPRE-GFP (Aldevron 5069-10), pALD-HELP (Aldevron 5082-10), AAV8 Rep-Cap Plasmid (GeneMedi P-RC09)
- Erlenmeyer shake flasks (e.g. Corning® 431143 or Thomson 931110)
- 10X Cell Lysis Buffer (e.g 500 mM Tris pH 8, 10% Tween® 20, 20 mM MgCl₂)
- 5 M Sodium Chloride (5 M NaCl)
- Benzonase® Nuclease (e.g. MilliporeSigma E1014 or equivalent)

AAV GENERATION IN SUSPENSION HEK 293 CELL CULTURES

NOTE: Use of the AAViator™ GMP Transfection System is only recommended for AAV production in suspension HEK 293 cell lines. For transfection of adherent HEK 293 cell lines, *TransIT-VirusGEN®* GMP Transfection Reagent is recommended.

Scale your AAV GMP run based on the **volume of complete growth medium** to be used as per **Table 1**. Examples of runs in commonly used bioreactor sizes are detailed in **Table 2**.

Table 1. Scaling worksheet for TransIT® AAViator GMP with RevIT™ GMP AAV Enhancer

Starting conditions per mL of complete growth medium					
	Per mL		Total culture volume		Reagent quantities
Complex Formation Solution	45.6 µL	×	_____ mL	=	_____ µL
Total Plasmid DNA (1 µg/µL stock)	1.5 µL	×		=	_____ µL
RevIT™ GMP AAV Enhancer	1.0 µL	×		=	_____ µL
TransIT® AAViator GMP Reagent	1.9 µL	×		=	_____ µL
Total Volume		50 µL			



Complete Mixing is paramount to achieving highest possible titers. DO NOT MIX during or after the DNA/transfection reagent incubation step.

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

Known compatible transfection complex formation solutions include the following;

- DMEM – high glucose (Dulbecco's Modified Eagle Medium) (MilliporeSigma D6429)
- Viral Production Medium (VPM) (Thermo Fisher A4817901)
- BalanCD HEK 293 (FujiFilm 91165)
- HyClone™ Peak Expression Medium (Cytiva SH31192)

Table 2. Scaling examples for transfection components

Examples					
	3 L	100 L	200 L	1000 L	
Complex Formation Solution	136.8 mL	4.56 L	9.12 L	45.6 L	
Total Plasmid DNA (1 µg/µL stock)	4.5 mL	150 mL	300 mL	1.5 L	
RevIT™ GMP AAV Enhancer	3 mL	100 mL	200 mL	1 L	
TransIT® AAViator GMP Reagent	5.7 mL	190 mL	380 mL	1.9 L	
Total Complex Volume	150 mL	5 L	10 L	50 L	

Transient Transfection Protocol

Volumes given below are **for a 30 mL culture**. Use **Table 1** to calculate the volumes required for your process or refer to **Table 2** for more examples.

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 $3 - 4 \times 10^6$ cells/mL the next day.
Perform cell counts and evaluate viability daily to ensure that cells are doubling every 24 hours and $\geq 95\%$ viable. 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% CO₂, shaking).

B. Prepare transfection complexes (immediately before transfection)

1. Warm TransIT® AAViator GMP Reagent and RevIT™ GMP AAV Enhancer to room temperature and vortex gently before using (see thaw time guidance in 'Before You Start').
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 1.368 mL of complex formation solution 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 45 µL of the DNA mixture prepared in Step B.4 to the tube containing complex formation solution. Mix completely.
6. Add 30 µL of RevIT™ GMP AAV Enhancer to the diluted DNA and basal culture medium. Mix completely.
7. Add 57 µL of TransIT® AAViator GMP Reagent to the diluted DNA:RevIT™ GMP mixture. Mix completely by inversion or vortexing. Do NOT agitate the transfection complexes again after this initial mixing.
8. Incubate at room temperature for 15-45 minutes without additional agitation to allow transfection complexes to form.

C. Distribute the complexes to cells in complete growth medium

1. Add the transfection 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 CO₂ levels (e.g. 37°C, 5-8% CO₂).
3. Incubate cultures for 48-72 hours prior to AAV harvest.

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® Nuclease (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 $4,100 \times g$ 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.







Do NOT allow the TransIT®-AAViator GMP 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 forming transfection complexes after the initial mixing. This will result in decreased titer.



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

Before You Start	Transfection Complex Formation	Incubation & Delivery
 <p>Passage cells 18-24 hours prior to transfection and ensure >95% viability.</p>  <p>Warm all reagents to room temperature. Before use, ensure each reagent is mixed to uniformity.</p> <p>Target VCD: 3×10^6 cells/mL</p>	<p>per mL of total culture, mix together in one sterile tube:</p> <p>Complex Formation Solution: 45.6 μL</p> <p>DNA: 1.0 μL + 1.5 μg (1)</p> <p>RevIT™ GMP: 0.5 μL + 1.5 μL (2)</p> <p>TransIT® AAViator GMP: 1.0 μL + 2.3 μL (3)</p> <p> Add TransIT® AAViator GMP to the mixture last and mix vigorously.</p>	 <p>Incubate complex for 15 - 45 minutes. Do not disturb.</p> <p>Transfer complex to cell culture and swirl to evenly distribute.</p> <p>Harvest AAV as needed, typically 48-72 hr post-transfection.</p> <p><i>Questions? Please contact Mirus Bio Technical Support!</i></p>

Collaborate with a scientist at Mirus Bio to design a transfection protocol optimized for your AAV manufacturing process. Connect at mirusbio.com/contact.

Forming Transfection Complexes at Large Scale

- Containers and tubing:**

- The transfection complex can be prepared using 2D bioprocessing bags or sterile transfer flasks and bottles. Ensure the container has enough headspace to allow for effective mixing.
- TransIT® AAViator GMP (solvent: EtOH) and RevIT™ GMP AAV Enhancer (solvent: DMSO) are compatible with containers and tubing made of high-density polyethylene (HDPE) and polypropylene (PP).
- DMSO and EtOH are known to be incompatible with hydroxy-terminated polyether (HTPE), polycarbonate (PC), polyethylene terephthalate glycol (PETG), polysulfone (PSU) and polyvinyl chloride (PVC).
- Avoid extended exposure of RevIT™ GMP AAV Enhancer to platinum-coated silicone tubing. For example, maintain RevIT™ GMP AAV Enhancer in sterile bottles until use. When ready to use, switch the cap to a filling/venting closure to pump RevIT™ GMP AAV Enhancer into diluted DNA mixtures or directly into cell culture.

- Filtering:**

If additional filtering is required for your process:

- Filter all components undiluted, i.e. "neat", before diluting them in an aqueous solution (e.g. DMEM). Do not filter transfection complexes once formed. The size of a transfection complex is larger than a 0.2 μ m pore size and so it will be filtered out.
- Known compatibility of filter materials for individual components is based on the reagent solvent (**Table 3**). Please check the filter manufacturer's specifications to ensure compatibility.

Table 3: Compatible filter materials

	PTFE Filter 0.2 μ m	Nylon Filter 0.2 μ m	PES Filter 0.2 μ m
TransIT® AAViator GMP Reagent	Unknown	Unknown	Compatible
RevIT™ GMP AAV Enhancer	Compatible	Compatible	Incompatible

- **Mixing:**
 - Add the DNA and RevIT™ GMP AAV Enhancer to basal culture media, mixing well after each addition (RevIT™ GMP AAV Enhancer can also be added directly to the cell culture at the time of transfection).
 - Add TransIT® AAViator GMP Reagent as the last component to the transfection mixture. If the current workflow established pre-dilution of the transfection reagent prior to combining it with the diluted DNA and RevIT™ mixture, do not allow the TransIT® AAViator GMP Reagent to incubate alone in aqueous solution > 5 min.
 - Mixing can be performed by inversion in a sterile vessel (10-20 times) or rocking gently on a rocking platform for 5-7 seconds (e.g. 25 rpm at a 12° angle).
 - After mixing, allow the transfection complexes to form by incubating stationary.
- **Transfer of the transfection complex:**
 - Near the end of the desired incubation time, begin gravity draining or pumping the transfection complexes into the bioreactor.
 - Complexes can be effectively pumped at speeds of 1-2 L/min through #73 tubing (3/8" inner diameter). If using a different size of tubing, adjust the flow rate accordingly.

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 <i>TransIT</i> ® Reagent: <i>RevIT</i> ™:DNA ratio	Determine the best <i>TransIT</i> ® AAViator GMP Reagent: <i>RevIT</i> ™ GMP AAV Enhancer:DNA ratio for each cell type. Titrate the <i>TransIT</i> ® AAViator GMP Reagent volume from 1-1.5 µL per 1 µg of DNA. Titrate the <i>RevIT</i> ™ 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 1.0-1.5 µg per 1 mL of culture. Start with 1.5 µg DNA per 1 mL of culture. Consider testing different amounts of DNA while scaling the amount of <i>TransIT</i> ® AAViator 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	Use a seeding strategy to ensure that 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.
<i>TransIT</i> ® AAViator GMP was not mixed properly	Warm <i>TransIT</i> ® AAViator GMP Reagent to room temperature and vortex gently before each use. If <i>TransIT</i> ® AAViator GMP Reagent is pre-diluted in complex formation solution, DNA should be added within 5 minutes. Incubating the <i>TransIT</i> ® AAViator GMP Reagent in complex formation solution alone for an extended time may result in reduced AAV yield.
<i>RevIT</i> ™ GMP AAV Enhancer was not prepared properly	Ensure that <i>RevIT</i> ™ GMP AAV Enhancer is completely thawed and vortexed gently prior to use.
Disruption of transfection complex formation	After initial mixing of DNA, <i>RevIT</i> ™ AAV Enhancer and <i>TransIT</i> ® AAViator 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, <i>TransIT</i> ® AAViator GMP Reagent, <i>RevIT</i> ™ 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 <i>not</i> 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 <i>Label IT</i> ® Nucleic Acid Labeling Kits to label the target plasmid or use Mirus pre-labeled <i>Label IT</i> ® Plasmid Delivery Controls (please refer to “Related Products” on Page 8). To verify efficient transfection, use <i>TransIT</i> ® AAViator GMP 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 cell density is too low at the time of transfection. For high virus titers using TransIT® AAViator GMP Reagent, ensure that HEK 293 suspension cell cultures are approximately 3×10^6 cells/mL at the time of transfection.
Cell morphology has changed	When generating AAV with RevIT™ 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.
Transfection complexes not evenly distributed after complex addition to cells	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.
	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.

RELATED PRODUCTS

- TransIT-VirusGEN® GMP Transfection Reagent
- RevIT™ GMP AAV Enhancer
- AAViator™ Upstream Production Kit

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



Reagent Agent®

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