AAViator™ Transfection System

Now with AAViator™ Transfection Complex Stabilizer

MIR 73750 and MIR 73745

SDS and Certificate of Analysis 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. 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.

TransIT® AAViator Transfection Reagent is intended to be used with *Rev*IT™ AAV Enhancer.

The AAViator™ Transfection System enables the generation of high titer AAV in HEK 293 cell types. Moreover, it allows for the acceleration of research and development while improving the full/empty capsid ratio and reducing the amount of plasmid DNA required per transfection.

The AAViator™ Transfection System includes:

- TransIT® AAViator Transfection Reagent proven for AAV production in suspension HEK 293 derived cells.
- *Rev*IT™ AAV Enhancer designed to increase AAV titer 2-4X for any serotype, reagent, HEK 293 cell line or media.
- AAViator™ Transfection Complex Stabilizer (AAViator™ Stabilizer) which allows for transfection volumes as low as 2% of total culture volume and increased transfection complexation time of up to 3 hours.

SPECIFICATIONS

Storage	Store TransIT® AAViator Transfection Reagent, AAViator™ Stabilizer and *Rev*IT™ AAV Enhancer at -10 to -30°C, tightly capped. ***Before each use***, warm to room temperature and vortex.
Stability / Guarantee	When properly stored and handled, TransIT® AAViator Transfection Reagent, AAViator™ Stabilizer and *Rev*IT™ AAV Enhancer are guaranteed for 6 months from date of purchase.

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

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

For Research Use Only

MATERIALS

The AAViator™ Transfection System is supplied in the following formats:

Product No.	TransIT® AAViator Transfection Reagent	*Rev*IT™ AAV Enhancer	AAViator™ Transfection Complex Stabilizer
MIR 73750	2 × 1.5 mL	1 × 1.5 mL	2 x 150 µL
MIR 73745	1 × 30 mL	10 × 1.5 mL	2 x 1.5 mL

BEFORE YOU START:

Important Tips for Optimal AAV Production

- **Cell culture conditions.** Culture suspension HEK 293 cells in appropriate complete growth medium, such as Cellvento 4HEK® (+ 6 mM 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 for suspension cells 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, use plasmid manufacturer recommendations or previously established ratios as a starting point.
    - Mirus recommends resuspending plasmids in water.
- **Ratio of TransIT® AAViator to DNA.** The optimal ratio between TransIT® AAViator Transfection Reagent and DNA is typically 1:1 to 1.5:1. The recommendation is to start at 1.25:1.
    - 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:

      TransIT® AAViator: 1 to 2.3 µL

      Total DNA: 1 to 1.5 µg
- ***Rev*IT™ AAV Enhancer.** Titrate *Rev*IT™ AAV Enhancer from 0.5 to 1.5 µL per 1 mL of culture. Use 1 µL per mL as a starting point. Thaw fully and vortex before use.

  *Rev*IT™ AAV Enhancer Thaw Time

	Room Temperature	37°C
*Rev*IT™ 1.5 mL	~4 hours	~30 minutes
*Rev*IT™ 200 mL	~48 hours	~5.5 hours

    - 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.
- **Complex formation conditions.** Prepare transfection complexes in compatible complex formation solution in a volume that is 5% or 2% of the total culture volume.
    - Mirus recommends a complex formation time of 20 minutes up to 3 hours when using the AAViator™ Stabilizer, but optimal formation time should be determined empirically.

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.

Preparing Stabilized TransIT® AAViator Reagent

AAViator™ Stabilizer must be added directly to TransIT® AAViator Transfection Reagent prior to preparing transfection complexes.

Supplement TransIT® AAViator Transfection Reagent with **0.08 to 0.12X by volume** of AAViator™ Stabilizer (**Table 1**). The optimal amount to add may depend on the DNA concentration, complex formation solution and cell line. Supplementing with 0.1X AAViator™ Stabilizer is an appropriate starting point.

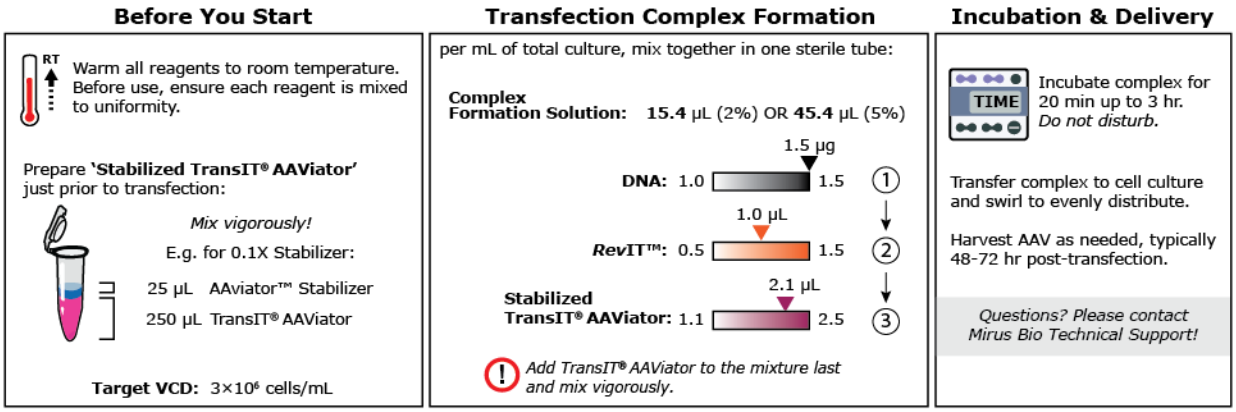
Table 1. Calculation worksheet for preparing Stabilized TransIT® AAViator

Volume of TransIT® AAViator Transfection Reagent		Concentration of Stabilizer (X)		Volume of AAViator™ Stabilizer to add	Total Volume
____ µL	×	0.__X	=	____ µL	____ µL
Example 450 µL	×	*0.1X*	=	*45 µL*	*495 µL*

1. Bring TransIT® AAViator and AAViator™ Stabilizer to room temperature, then vortex to ensure homogeneity.
2. Determine volumes of TransIT® AAViator and AAViator™ Stabilizer required for transfection (**Table 1**). Typically, the optimal amount of AAViator™ Stabilizer is between 0.08X to 0.12X by volume.
3. Place _____ µL of TransIT® AAViator in a non-treated polypropylene sterile tube (e.g. MidSci #MID15C).
4. Add _____ µL of AAViator™ Stabilizer to the tube. Vortex to mix.
5. This mixture is referred to as the 'Stabilized TransIT® AAViator Reagent' and may be used immediately after preparation or stored at -10 to -30°C for 1 week, tightly capped.

NOTE: When using Stabilized TransIT® AAViator Reagent for transfection, be sure to account for the additional volume that has been added during its preparation.

- For example, if 1.9 µL of TransIT® AAViator Reagent (non-stabilized) is typically used to form transfection complexes, then 2.1 µL of 0.1X Stabilized TransIT® AAViator Reagent should be used.
- If using another concentration of AAViator™ Stabilizer and/or volume of TransIT® AAViator, multiply 1.__X (concentration of AAViator™ Stabilizer) by the volume of TransIT® AAViator to calculate the correct volume.
  E.g. 1.08 x 2.5 µL = 2.7 µL of 0.08X Stabilized TransIT® AAViator *per mL of total culture volume.*



Wondering what a transfection complex is?
Learn about it at mirusbio.com/deliverance

Materials Required, But Not Supplied

- Suspension HEK 293 Cells (e.g. Viral Production Cells 2.0, Gibco A49784)
- Complete Culture Medium (e.g. Cellvento 4HEK® (MilliporeSigma 125193). Cellvento 4HEK® requires supplementation with 6mM L-glutamine (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. Thomson 931110)
- 10X Cell Lysis Buffer (500 mM Tris pH 8, 10% Tween® 20, 20 mM MgCl2)
- 5 M Sodium Chloride (5 M NaCl)
- Benzonase® Nuclease (e.g. MilliporeSigma E1014)

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 30 mL of complete growth medium. If using an alternate cell culture vessel, scale based on the **volume of complete growth medium** to be used as per **Table 2** (below).

Table 2. Scaling worksheet for TransIT® AAViator with *Rev*IT™ AAV Enhancer at 2% or 5% of total culture volume

Starting conditions per mL of complete growth medium						
	2% **OR**	5%		Total culture volume		Reagent quantities
Complex Formation Solution	15.4 µL	45.4 µL	×	_____ mL	=	_____ µL
Total Plasmid DNA (1 µg/µL stock)	1.5 µL	1.5 µL	×		=	_____ µL
*Rev*IT™ AAV Enhancer	1.0 µL	1.0 µL	×		=	_____ µL
0.1X Stabilized TransIT® AAViator Reagent	2.1 µL	2.1 µL	×		=	_____ µL
Total Volume	**20 µL**	**50 µL**				

If using more than 1.5 µg of DNA per mL of total cell culture, using a 3% complex formation volume yields better results.

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

Complex Formation Solution

Complex formation solution is the media with which plasmids and transfection reagent are mixed prior to adding to the cell culture. Known compatible complex formation solutions include:

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

Optimization

Mirus recommends forming complexes in a volume that is 2% of total culture volume (i.e. 600 µL for a 30 mL culture), when utilizing AAViator™ Stabilizer, and a complex formation time of up to 3 hours. During optimization, the most appropriate control is an 'unstabilized' transfection complex performed at 5% of total culture volume with a 20 minute complex formation time.

In addition to these conditions, testing concentrations of AAViator™ Stabilizer between 0.08X and 0.12X is also recommended.

If using 2 µg of DNA per mL of cell culture, *Mirus R&D found that yields are better if using a 3% complex, rather than 2%. Adjust the volume of complex formation solution accordingly i.e total complex volume will be 30 µL instead of 20 µL, per mL of cell culture.*

Transient Transfection Protocol for 30 mL cell culture at a 2% complex volume

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.
   Perform cell counts and evaluate viability daily to ensure that cells are doubling every 24 hours and ≥ 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% CO_2, shaking).

B. Prepare transfection complexes (immediately before transfection)

1. Prepare Stabilized TransIT® AAViator as per Table 1.
2. Warm all reagents to room temperature and vortex gently before using.
3. 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).
4. Place 462 µL of complex formation solution in a sterile tube.
5. In a separate sterile tube, combine AAV plasmids per manufacturer recommendations to a final concentration of 1 µg/µL. Mix thoroughly.
6. Transfer 45 µL of the DNA mixture prepared in Step B.5 to the tube containing complex formation solution. Vortex with a 0.5-second pulse.
7. Add 30 µL of *Rev*IT™ AAV Enhancer to the diluted DNA and complex formation solution. Vortex with a 0.5-second pulse.
8. Add 63 µL of 0.1X Stabilized TransIT® AAViator Reagent to the diluted DNA:*Rev*IT™ mixture. Mix completely by vortexing for three 0.5-second pulses. Do NOT agitate transfection complexes again after this initial mixing.
9. Incubate at room temperature for 20 minutes up to 3 hours without additional agitation to allow transfection complexes to form.

Add TransIT® AAViator to the transfection mixture last for best results.

Do NOT agitate transfection complexes after the initial mixing. This will result in decreased titer.

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 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. ~31.5 mL) to a sterile conical tube or appropriate vessel.
2. Add 0.1X volume of 10X Cell Lysis Buffer (i.e. 3.2 mL) and 100 U/mL Benzonase® (i.e. 3,200 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. 3.2 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.

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 size of the container has enough headspace to allow for effective mixing.
  - TransIT® AAViator (solvent - EtOH) and *Rev*IT™ AAV Enhancer (solvent - DMSO) are compatible with containers and tubing made of high-density polyethylene (HDPE) and polypropylene (PP).
  - DMSO and ethanol 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 platinum-coated silicon tubing to *Rev*IT™ AAV Enhancer. For example, maintain *Rev*IT™ AAV Enhancer in sterile bottles and protected from light until use. When ready to use, then switch the cap to a filling/venting closure to pump *Rev*IT™ 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 they have been diluted in an aqueous solution e.g. DMEM.
  - **Table 2** below shows known compatibility for filter materials.

  Table 2. Compatible filter materials

	PTFE 0.2 µm	Nylon 0.2 µm	PES 0.2 µm
TransIT® AAViator Reagent	*Unknown*	*Unknown*	Compatible
*Rev*IT™ AAV Enhancer	Compatible	Compatible	Incompatible
AAViator™ Stabilizer	Compatible	*Unknown*	*Unknown*

  - Do not filter transfection complexes once formed. The size of a transfection complex is larger than a 0.2 µm pore size and so will be filtered out.
- **Mixing:**
  - Add the DNA and *Rev*IT™ AAV Enhancer to complex formation solution, mixing well after each addition. (*Rev*IT™ AAV Enhancer can also be added directly to the cell culture at the time of transfection.)
  - Add TransIT® AAViator Reagent as the last component to the transfection mixture. Do not allow the TransIT® AAViator Reagent to incubate alone in aqueous solution > 5 minutes.
  - Mixing can be performed by inversion in a sterile vessel (10-20 times) or rocking gently on a rocking platform for 5-30 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 TransIT® Reagent:*Rev*IT™:DNA ratio	Determine the best TransIT® AAViator Reagent:*Rev*IT™ AAV Enhancer:DNA ratio for each cell type. Titrate the TransIT® AAViator Reagent volume from 1-1.5 µL per 1 µg of DNA. Titrate the *Rev*IT™ 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 TransIT® AAViator accordingly.
Suboptimal complex formation conditions	Ensure a compatible complex formation solution is being used. These can be found on Page 4.
	If using a DNA mass higher than 1.5 µg per mL of cell culture, yields are typically higher when using a 3% complex formation volume, rather than 2%. (i.e. amount of complex formation solution as a percentage of the total cell culture volume).
Suboptimal AAViator™ Stabilizer concentration	Determine the optimal amount of AAViator™ Stabilizer empirically by testing different concentrations (0.08X, 0.1X and 0.12X). The optimal amount may depend on the DNA concentration, complex formation solution and cell line. 0.1X is an appropriate starting point.
Incorrect AAViator™ Stabilizer addition	AAViator™ Stabilizer must be added directly to TransIT® AAViator Transfection Reagent and mixed well prior to beginning the transfection process.
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.
TransIT® AAViator was not mixed properly	Warm TransIT® AAViator Reagent to room temperature and vortex gently before each use.
	If TransIT® AAViator Reagent is pre-diluted in complex formation solution, DNA should be added within 5 minutes. Incubating the TransIT® AAViator 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™ AAV Enhancer and TransIT® AAViator 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, TransIT® AAViator Reagent, *Rev*IT™ 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® Nucleic Acid Labeling Kits to label the target plasmid or use Mirus prelabeled *Label* IT® Plasmid Delivery Controls (please refer to "Related Products" on Page 8).
	To verify efficient transfection, use TransIT® AAViator Reagent to deliver a positive control such as a luciferase, beta-galactosidase or green fluorescent protein (GFP) encoding plasmid.

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 TransIT® AAViator Reagent, ensure that cell cultures are approximately 3 × 10^6 cells/mL at the time of transfection.
Cell morphology has changed	When generating AAV with *Rev*IT™ 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 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.

RELATED PRODUCTS

- AAViator™ Production Platform
- *Trans*IT-VirusGEN® GMP Transfection Reagent
- *Trans*IT-VirusGEN® Transfection Reagent
- *Rev*IT™ GMP AAV Enhancer
- VirusGEN® GMP AAV Transfection Kit
- VirusGEN® AAV Transfection Kit
- *Label* IT® Nucleic Acid Labeling Kits

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

Reagent Agent®

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EMPOWER Discovery
IMPROVE Life