VirusGEN® AAV Transfection Kit with *Rev*IT™ AAV Enhancer

Now with VirusGEN® Transfection Complex Stabilizer

MIR 8007 and MIR 8008

Quick Reference Protocol, CoA and SDS available at mirusbio.com/literature

INTRODUCTION

Transient transfection of suspension human embryonic kidney (HEK) 293 cells is a flexible method for manufacturing recombinant adeno-associated virus (AAV). This process involves mixing and incubating recombinant AAV plasmid DNA with a transfection reagent to form DNA-reagent complexes, which are then delivered to HEK 293 cells to initiate AAV production. Typically, the process is time-limited because the DNA-reagent complexes will grow and exceed an effective size over time. The VirusGEN® AAV Transfection Kit with *Rev*IT™ AAV Enhancer, now with VirusGEN® Transfection Complex Stabilizer, overcomes this limitation by enabling extended complex formation time and concentrated DNA-reagent complexation conditions.

When supplemented with the VirusGEN® Transfection Complex Stabilizer, the *Trans*IT-VirusGEN® Transfection Reagent can be complexed with DNA in volumes as low as 2% of the total cell culture volume. These complexes retain comparable efficacy whether incubated for 30 minutes or 3 hours, simplifying transfection for high-throughput and large-scale applications. Additionally, the *Rev*IT™ AAV Enhancer synergizes with the transfection reagent to further boost productivity, resulting in high titers of recombinant AAV with full-length vector genomes. All kit components are chemically defined and support robust AAV production across diverse HEK 293 cell lines and media and AAV serotypes.

SPECIFICATIONS

Storage	Store *Trans*IT-VirusGEN® Transfection Reagent, VirusGEN® Transfection Complex Stabilizer and *Rev*IT™ AAV Enhancer at -10 to -30°C, tightly capped. *Rev*IT™ AAV Enhancer is known to maintain function through at least five freeze-thaw cycles (thawed in a 37°C incubator).
Stability / Guarantee	When properly stored and handled, *Trans*IT-VirusGEN® Transfection Reagent is guaranteed for 1 year from date of purchase. VirusGEN® Transfection Complex Stabilizer is guaranteed for 3 months from date of purchase. *Rev*IT™ AAV Enhancer is 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 < 19°C. A 1.5 mL vial will require ~4 hours to thaw at room temperature or ~30 minutes at 37°C.

MATERIALS

Product No.	Volume of *Trans*IT-VirusGEN® Transfection Reagent	Volume of VirusGEN® Transfection Complex Stabilizer	Volume of *Rev*IT™ AAV Enhancer
MIR 8007	2 × 1.5 mL	2 × 150 µL	1 × 1.5 mL
MIR 8008	1 × 30 mL	2 × 1.5 mL	10 × 1.5 mL

For Research Use Only

Materials Required, But Not Supplied

Material	Example (Manufacturer Cat. No.)
Suspension HEK 293 Cells	Viral Production Cells 2.0 (Gibco A49784)
Complete Cell Culture Media	Cellvento® 4HEK Medium (MilliporeSigma 1251931000) with 6 mM L-Glutamine (MilliporeSigma 59202C)
Complex Formation Solution	Phosphate Buffered Saline (PBS) (MilliporeSigma D8537)
Plasmid DNA	pALD-ITR-WPRE-GFP (Aldevron 5069-10) pALD-HELP (Aldevron 5082-10) AAV8 Rep-Cap Plasmid (GeneMedi P-RC09)
Erlenmeyer Shake Flasks	Optimum Growth Flask (Thomson 931110)
10X Cell Lysis Buffer	500 mM Tris pH 8, 10% Tween® 20, 20 mM $MgCl_2$
5 M Sodium Chloride	5 M NaCl
DNA/RNA Nuclease	Benzonase® Nuclease (MilliporeSigma E1014)

BEFORE YOU START

Important Tips for Optimal AAV Production

Cell culture conditions:

- This protocol describes AAV production in suspension HEK 293 cell lines. Please contact Mirus Bio Technical Support for optimization in adherent cell cultures.
- Ensure cells are ≥ 95% viable and doubling as expected before transfection.
- Typically, optimal viable cell density (VCD) is 3×10^6 cells/mL at transfection.
- After transfection, an exchange of cell culture medium is *not* necessary.

AAV packaging and transfer plasmids:

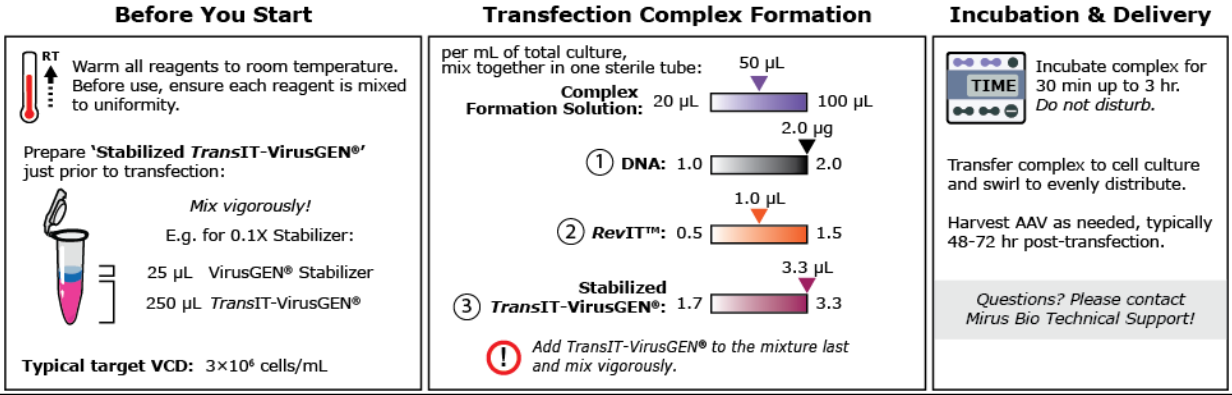
- The optimal ratio between plasmids will depend on the vector backbone and genes being expressed.
- Mirus recommends resuspending plasmids in water.
- Use plasmid manufacturer recommendations or previously established ratios as a starting point for optimization.

Complex formation solution:

- The media used to mix DNA with transfection reagent (i.e. complex formation solution) can affect resultant AAV yield, as components of the media may affect the complexation of transfection reagent and DNA.
- Known compatible complex formation solutions: PBS, Viral Production Medium (Thermo Fisher), BalanCD HEK293 (FujiFilm), HyClone™ Peak Expression Medium (Cytiva).
- If not listed above, Mirus recommends empirically determining whether your preferred complex formation solution/basal media is compatible.

Container and tubing material:

- *Trans*IT-VirusGEN® (solvent – ethanol (EtOH)), VirusGEN® Stabilizer (solvent – EtOH) and *Rev*IT™ AAV Enhancer (solvent – DMSO) are known to be compatible with high-density polyethylene (HDPE) and polypropylene (PP) containers and tubing.
- Known incompatibility with DMSO/EtOH: hydroxy-terminated polyether (HTPE), polycarbonate (PC), polyethylene terephthalate glycol (PETG), polysulfone (PSU), polyvinyl chloride (PVC) and polystyrene (PS).
- All materials should be tested for compatibility.



Preparing Stabilized *Trans*IT-VirusGEN® Reagent

VirusGEN® Transfection Complex Stabilizer (VirusGEN® Stabilizer) must be added directly to *Trans*IT-VirusGEN® Transfection Reagent prior to preparing transfection complexes.

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

Table 1. Calculation worksheet for preparing Stabilized *Trans*IT-VirusGEN®

Volume of *Trans*IT-VirusGEN® Transfection Reagent		Concentration of Stabilizer (X)		Volume of VirusGEN® Stabilizer to add	Total Volume
____ µL	×	0.__X	=	____ µL	____ µL
Example 450 µL	×	*0.1X*	=	*45 µL*	*495 µL*

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

NOTE: When using Stabilized *Trans*IT-VirusGEN® Reagent for transfection, be sure to account for the additional volume that has been added during its preparation.

- For example, if 3 µL of *Trans*IT-VirusGEN® Reagent (non-stabilized) is typically used to form transfection complexes, then 3.3 µL of 0.1X Stabilized *Trans*IT-VirusGEN® Reagent should be used.
- If using another concentration of VirusGEN® Stabilizer and/or volume of *Trans*IT-VirusGEN®, multiply 1.__X (concentration of VirusGEN® Stabilizer) by the volume of *Trans*IT-VirusGEN® to calculate the correct volume. *E.g. 1.08 x 2.5 µL = 2.7 µL of 0.08X Stabilized TransIT-VirusGEN® per mL of total culture volume.*

TRANSIENT TRANSFECTION PROTOCOL

The following procedure describes transfection of HEK 293 cells cultured in 30 mL of media in a 125 mL Erlenmeyer shake flask using recommended starting conditions. If using a different culture volume, refer to **Table 2** to calculate required reagent quantities.

Table 2. Calculation worksheet for scaling complexes at 2% or 5% of total cell culture volume

Starting conditions per mL of cell culture							
	2%	**OR** **5%**		**Total Culture Volume**		**Reagent Quantities**	
Complex Formation Solution	13.7 µL	43.7 µL	×		=		µL
Total Plasmid DNA (1 µg/µL stock)	2.0 µL	2.0 µL	×		=		µL
*Rev*IT™ AAV Enhancer	1.0 µL	1.0 µL	×	mL	=		µL
0.1X Stabilized *Trans*IT-VirusGEN® Reagent	3.3 µL	3.3 µL	×		=		µL
Total Complex Volume	**20 µL**	**50 µL**					

Mix packaging and transfer plasmids together prior to use in transfection.

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

For example, premix AAV packaging and transfer plasmids to a stock solution of 1 µg/µL and add 2 µL to deliver 2 µg of Total Plasmid DNA.

Transfection Protocol per 30 mL HEK 293 Culture

A. Maintain HEK 293 cells

1. Passage suspension HEK 293 cells prior to transfection to ensure that they are actively dividing and to obtain a density of 3 - 4×10^6 cells/mL at the time of transfection.
2. Incubate cells under appropriate conditions (e.g. 37°C, 5-8% CO_2, shaking).
3. Perform cell counts and evaluate viability daily to ensure that cells are doubling every ≤ 24 hours and are ≥ 95% viable. Do not proceed with transfection if cell growth or viability is lower than expected.

B. Prepare DNA : *Rev*IT™ AAV Enhancer : Stabilized *Trans*IT-VirusGEN® complexes

1. Prepare Stabilized *Trans*IT-VirusGEN® as per Table 1.
2. Warm all reagents to room temperature and vortex gently before using.

   NOTE: If thawing *Rev*IT™ AAV Enhancer 1.5mL at room temperature, allow ~4 hours and ensure ambient air temperature is > 19°C. Thawing can be accelerated to ~30 minutes by incubating at 37°C.

3. Immediately prior to transfection, seed 30 mL of HEK 293 cells to a viable cell density of 3×10^6 cells/mL in an appropriate vessel (e.g. 30mL into a 125mL Erlenmeyer shake flask).
4. In one sterile tube, add in the following order:

		Required Complex Volume (% of Total Culture Volume)		
		2%	**or**	5%
i.	Complex Formation Solution	411 µL		1.311 mL
ii.	Total Plasmid DNA (1 µg/µL stock)	60 µL		60 µL
iii.	*Rev*IT™ AAV Enhancer	30 µL		30 µL
iv.	0.1X Stabilized *Trans*IT-VirusGEN® Reagent	99 µL		99 µL

   Mix well after *each* addition to ensure uniform transfection complex formation.

   WARNING! Avoid pre-diluting *Trans*IT-VirusGEN® Reagent in media or buffer. Add *last* for best results.

5. Allow the transfection complex to form without further agitation/vortexing for 30 minutes up to 3 hours. Do not agitate or vortex during or after the incubation period.

C. Distribute the complexes to cells in complete growth medium

1. Add the DNA : *Rev*IT™ : Stabilized *Trans*IT-VirusGEN® complexes to the shake flask, swirling gently to distribute.
2. Incubate cultures until harvesting AAV, typically 48-72 hours post-transfection.

D. Harvest and store AAV

1. Following the 48-72 hour incubation, transfer the entire cell culture to a sterile conical tube.
2. Add 0.1X volume of 10X Cell Lysis Buffer (e.g. 3.15 mL for 31.5 mL culture) and 100 U/mL Benzonase® Nuclease (e.g. 3,150 U for 31.5 mL culture). Mix completely and incubate at 37°C for 1.5 hours with shaking.
3. Add 0.1X volume of 5 M NaCl (e.g. 3.15 mL for 31.5 mL culture) 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 supernatant containing AAV to a new sterile tube.
5. Store AAV stocks at -80°C.

See 'Preparing Stabilized *Trans*IT-VirusGEN®' on Page 3.

Mix well after addition of *each* component to ensure uniform transfection complex formation. If vortexing, a "tornado" should form in the tube.

*Trans*IT-VirusGEN® Reagent should not incubate alone in aqueous solutions for > 5 minutes. Add *Trans*IT-VirusGEN® last for optimal complex formation.

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.
- *Trans*IT-VirusGEN® (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 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.
- Avoid tubes made of polystyrene (PS) and tubes with coatings, such as low-protein binding tubes.

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. PBS. **Table 3** shows known compatibility for filter materials.

Table 3: Compatible filter materials.

	PTFE Filter 0.22 µm	Nylon Filter 0.2 µm	PES Filter 0.2 µm
*Trans*IT-VirusGEN® Reagent	*Unknown*	*Unknown*	Compatible
*Rev*IT™ AAV Enhancer	Compatible	Compatible	Incompatible
VirusGEN® Transfection Complex Stabilizer	Compatible	*Unknown*	*Unknown*

- Do not filter DNA : *Rev*IT™ : *Trans*IT-VirusGEN® complexes once formed. The size of complexes is typically > 0.2 µm 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 *Trans*IT-VirusGEN® Reagent as the last component to the transfection mixture. Do not allow the *Trans*IT-VirusGEN® Reagent to incubate alone in aqueous solution > 5 minutes.
- Mixing can be performed by inversion (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.

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

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® Reagent : *Rev*IT™ AAV Enhancer : DNA ratio for each cell type. Titrate the *Trans*IT-VirusGEN® Reagent volume from 1-3 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 VirusGEN® Stabilizer Concentration	Determine the optimal concentration of VirusGEN® Transfection Complex Stabilizer for your process by testing different concentrations (e.g. 0.08X, 0.1X or 0.12X) as described on Page 3. Supplementing the transfection reagent with too much or too little may reduce resultant titer.
	The optimal amount to add may depend on the DNA concentration, complex formation solution and cell line that are used in transfection. Supplementing with 0.1X VirusGEN® Stabilizer is an appropriate starting point.
Incorrect addition of VirusGEN® Stabilizer	VirusGEN® Transfection Complex Stabilizer must be added directly to *Trans*IT-VirusGEN® Transfection Reagent prior to using the reagent in transfection. After supplementing the transfection reagent with VirusGEN® Transfection Complex Stabilizer, mix well by vortexing.
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® accordingly.
Low-quality plasmid DNA	Use highly purified, sterile, endotoxin- and contaminant-free DNA for transfection.
	We recommend using MiraCLEAN® Endotoxin Removal Kit for removal of endotoxin from your DNA preparation. See 'related products' on Page 7. Alternatively, 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.
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® 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 minutes. Incubating the *Trans*IT-VirusGEN® Reagent in Complex Formation Solution alone for an extended time typically results in reduced production of functional virus.
Disruption of transfection complex formation	After initial mixing of DNA, *Rev*IT™ AAV Enhancer and Stabilized *Trans*IT-VirusGEN® Reagent, do not agitate the complexes again during or after the incubation period, e.g. do not vortex or invert before adding to cultures.
Precipitate formation during transfection complex formation	During complex formation, scale all reagents appropriately. See **Table 2.** Calculation worksheet for scaling complexes at 2% or 5% of total cell culture volume on Page 3.
	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, provided the complexes are well mixed.
Verify Transfection Efficiency	To assess delivery efficiency of plasmid DNA, use *Label* IT® Nucleic Acid Labeling Kits to label the target plasmid or prelabeled *Label* IT® Plasmid Delivery Controls. See 'related products' on Page 7.
	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 3×10^6 cells/mL (for suspension cell transfections) 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 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.

RELATED PRODUCTS

- *Trans*IT-VirusGEN® GMP Transfection Reagent
- *Rev*IT™ GMP AAV Enhancer
- VirusGEN® GMP LV Transfection Kit
- MiraCLEAN® Endotoxin Removal Kit
- *Label* IT® Plasmid Delivery Controls
- *Label* IT® Nucleic Acid Labeling Kits
- Ingenio® Electroporation Solution and Kits

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