*Trans*IT-VirusGEN® GMP Transfection Reagent

Protocol for MIR 6845-GMP

SDS available at mirusbio.com/6845

INTRODUCTION

Efficient, high titer and large-scale viral vector manufacturing processes are necessary for production of viral vectors such as lentivirus and adeno-associated virus (AAV) for gene- and cell-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 both needs by providing robust titers for lentivirus and AAV production and comprehensive quality documentation for researchers developing biotherapeutics.

*Trans*IT-VirusGEN® GMP is identical in formulation to the *Trans*IT-VirusGEN® Transfection Reagent. By screening lipid and polymer libraries to identify a reagent formulation that enhances delivery of virus packaging and transfer vectors to HEK 293 cells, Mirus scientists identified this novel transfection formulation that enables high titer lentivirus and AAV production in both adherent, serum-containing cultures, as well as serum-free suspension HEK 293-derived cell types. The streamlined virus generation workflows and culture format versatility make *Trans*IT-VirusGEN® an ideal reagent for scientists utilizing a variety of virus platforms. With *Trans*IT-VirusGEN® GMP, researchers can expect the same reliable, scalable and flexible performance of the *Trans*IT-VirusGEN® Transfection Reagent as they progress from discovery and development to clinical trials and commercial manufacturing.

The *Trans*IT-VirusGEN® GMP Transfection Reagent undergoes release testing for appearance, sterility, formulation identity, mycoplasma and endotoxin.

SPECIFICATIONS

Storage	Store *Trans*IT-VirusGEN® GMP Reagent at -10 to -30°C, tightly capped. ***Before each use***, warm to room temperature and vortex gently.
Stability / Guarantee	Guaranteed as noted on the Certificate of Analysis when properly stored and handled.

Warm *Trans*IT-VirusGEN® GMP Reagent to room temperature and vortex gently before each use.

MATERIALS

Materials Supplied

*Trans*IT-VirusGEN® GMP Transfection Reagent is supplied in the following format.

Product No.	Quantity
MIR 6845-GMP	1 × 150 ml

For Materials Required but Not Supplied, See Protocol Sections:

- (I) Lentivirus Generation in Adherent HEK 293 Cell Cultures
- (II) Lentivirus Generation in Suspension HEK 293 Cell Cultures
- (III) Lentivirus Transduction and Titering Protocol Using GFP Reporter Virus
- (IV) AAV Generation in Adherent HEK 293 Cell Cultures
- (V) AAV Generation in Suspension HEK 293 Cell Cultures
- (VI) AAV Transduction/Titering Method Using a GFP Reporter Virus

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

BEFORE YOU START:

Important Tips for Optimal Lentivirus or AAV Production

The suggestions below yield high efficiency plasmid DNA transfection using the *Trans*IT-VirusGEN® GMP Transfection Reagent.

- **Cell culture conditions.** Culture cells in the appropriate medium, with or without serum (e.g. DMEM + 10% FBS + 10 mM HEPES pH 7.4 for adherent HEK 293 cultures; BalanCD HEK293 for suspension HEK 293 cultures). 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 (% confluence) at transfection.** The recommended cell density for adherent HEK 293 cells is 80-95% confluence at the time of transfection. The recommended cell density for suspension HEK 293 cells is 2 - 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.
- **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.
- **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 2-4 µl (lentivirus) or 1-3 µl (AAV) per 1 µg total DNA. Refer to **Tables 1-4** in the Lentivirus and AAV sections for recommended starting conditions based on cell culture vessel size.
- **Complex formation conditions.** Prepare *Trans*IT-VirusGEN® GMP Reagent:DNA complexes in PBS or compatible basal cell culture media in a volume that is 5-10% of the total culture volume. For each unique vector construct, we recommend evaluating complex formation times between five minutes and one hour to identify an optimal time for maximal viral titer and quality. As a starting point, we recommend a complex formation time of 15-30 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.

SECTION I: Lentivirus Generation in Adherent HEK 293 Cell Cultures

The following procedure describes plasmid DNA transfections for lentivirus generation in adherent HEK 293 cell types in a 6-well plate format. The surface areas of other culture vessels are different, and transfections must be scaled accordingly. Appropriately increase or decrease the amounts of serum-free medium, *Trans*IT-VirusGEN® GMP Reagent, total plasmid DNA and complete culture medium based on the size of the cell culture vessel (refer to **Table 1** below).

Table 1. Recommended *Trans*IT-VirusGEN® GMP starting conditions for LV production

Culture vessel	6-well plate	10-cm dish	T75 flask	T175 flask	Corning® 2-STACK	Corning® 5-STACK
Surface area	9.6 cm^2	59 cm^2	75 cm^2	175 cm^2	1272 cm^2	3180 cm^2
Complete growth medium	2 ml	10 ml	15 ml	35 ml	260 ml	650 ml
PBS or Serum-free medium	200 µl	1.0 ml	1.5 ml	3.5 ml	26 ml	65 ml
Total Plasmid DNA (1 µg/µl stock)	2 µl	10 µl	15 µl	35 µl	260 µl	650 µl
*Trans*IT-VirusGEN® GMP Reagent	6 µl	30 µl	45 µl	105 µl	780 µl	1.95 ml

NOTE: Total plasmid DNA refers to the combined weight of transfer and packaging plasmids (in µg) per transfection.

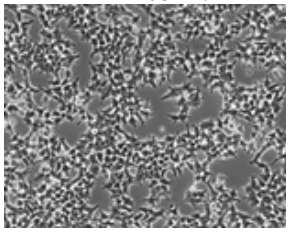
Surface areas are based on Falcon plates, dishes and flasks, and Corning CellSTACK® Culture Chambers. Volumes are per well (or dish) for a given culture vessel. For vessels not listed in this table, volumes of PBS, total DNA and *Trans*IT-VirusGEN® GMP Reagent can typically be scaled according to surface area (cm^2).

Materials Required but Not Supplied

- HEK 293 cells (e.g. HEK 293T/17 cells, ATCC Cat. No. CRL-11268)
- Complete culture medium (e.g. DMEM + 10% FBS + 10 mM HEPES pH 7.4)
- Plasmid DNA (e.g. pALD-Lenti System, Aldevron Cat. No. pALD-Lenti/VSV-G/GagPol/Rev)
- Phosphate Buffered Saline (PBS) (e.g. MilliporeSigma Cat. No. D8537)
- 0.45 µm PVDF filter (e.g. Millipore Cat. No. SE1M003M00 or SLHV033RS)
- Reporter assay as required

Passage cultured cells 18-24 hours before transfection to ensure active cell division at the time of transfection.

Representative image of ~80% confluent HEK 293T/17 cells:



Transient Plasmid Transfection Protocol per Well of a 6-Well Plate

A. Plate cells

1. Approximately 18-24 hours before transfection, plate cells in 2.0 ml complete growth medium per well in a 6-well plate. A starting cell density of 4.0 - 5.0 × 10^5 cells/ml is recommended. Cultures should be 80-95% confluent at the time of transfection (see representative image at right).
2. Incubate cell cultures at 37°C in 5% CO_2 overnight.

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

1. Warm *Trans*IT-VirusGEN® GMP Transfection Reagent to room temperature and vortex gently before using.
2. Place 200 µl of PBS in a sterile tube.
3. In a separate sterile tube, combine the packaging plasmid premix (or individual plasmids) and transfer plasmid encoding the gene-of-interest. Mix thoroughly.
4. Transfer 2 µg of the total plasmid DNA prepared in Step B.3 to the tube containing PBS. Mix completely.
5. Add 6 µl of *Trans*IT-VirusGEN® GMP Reagent to the diluted DNA mixture. Mix completely by inversion or vortexing. Do NOT agitate Reagent:DNA complexes again after this initial mixing.
   NOTE: This is a 3:1 mixture of transfection reagent to total DNA (vol:wt), which can be further optimized for lentivirus production using *Trans*IT-VirusGEN® GMP Reagent.

Do NOT allow the *Trans*IT-VirusGEN® GMP 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.

6. Incubate the mixture at room temperature for 15-30 minutes stationary.
   NOTE: The ideal complex formation time may depend on the vector production platform but is typically between 5-60 minutes.

C. Distribute the complexes to cells in complete growth medium

1. Add the *Trans*IT-VirusGEN® GMP Reagent:DNA complexes (prepared in Step B) drop-wise to different areas of the wells.
2. Gently rock the culture vessel back-and-forth and from side-to-side to evenly distribute the *Trans*IT-VirusGEN® GMP Reagent:DNA complexes.
3. Incubate at 37°C in 5% CO_2 for 48 hours.
   NOTE: It is not necessary to replace the complete growth medium with fresh medium post-transfection.

There is no need to change culture medium after transfection.

Transfection complexes, visualized as small particles, are sometimes observed following transfection. The complexes are not toxic to cells and do not affect transfection efficiency or transgene expression.

D. Harvest and storage of lentivirus

1. Harvest cell supernatant containing recombinant lentivirus particles.
   NOTE: If cells detach during harvest, centrifuge cells at 300 × g for 5 minutes and retain the virus-containing supernatant.
2. Filter virus-containing supernatant through a 0.45 µm PVDF filter to remove any cells.
3. Immediately flash freeze aliquots of lentivirus in cryogenic tubes and store at -80°C.

SECTION II: Lentivirus Generation in Suspension HEK 293 Cell Cultures

The following procedure describes plasmid DNA transfections for lentivirus generation in 125 ml Erlenmeyer shake flasks using 25 ml of complete growth medium. If using alternate cell culture vessels, increase or decrease the amounts of serum-free complex medium, *Trans*IT-VirusGEN® GMP 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 2** (below).

We recommend premixing the packaging and transfer plasmids. For each unique construct, empirically determine and use the optimal ratio between plasmids for best results. Use plasmid manufacturer recommendations or previously established ratios as a starting point.

Table 2. Scaling worksheet for *Trans*IT-VirusGEN® GMP Reagent for LV transfections

Starting conditions per milliliter of complete growth medium (Lentivirus Generation)							
	Per 1 ml			Total culture volume		Reagent quantities	
PBS or serum-free medium	0.1	ml	×	_____	ml =	_____	ml
Total plasmid DNA (1 µg/µl stock)	1	µl	×	_____	ml =	_____	µl
*Trans*IT-VirusGEN® GMP Reagent	3	µl	×	_____	ml =	_____	µl

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

Materials Required but Not Supplied

- Suspension HEK 293 Cells (e.g. Viral Production Cells, Gibco Cat. No. A35347)
- Complete Culture Medium (e.g. LV-MAX™ Production Medium (Gibco Cat. No. A3583401) or BalanCD HEK293 (Irvine Scientific Cat. No. 91165))
- Plasmid DNA (e.g. pALD-Lenti System, Aldevron Cat. No. pALD-Lenti/VSV-G/GagPol/Rev)
- Phosphate Buffered Saline (PBS) (e.g. MilliporeSigma Cat. No. D8537)
- Erlenmeyer shake flasks (e.g. Corning® Cat. No. 431143 or Thomson Cat. No. 931110)
- 0.45 µm PVDF filter (e.g. Millipore Cat. No. SE1M003M00 or SLHV033RS)
- 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 - 3 × 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 are ≥ 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).

Passage 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 - 3 × 10^6 cells/ml into a transfection culture vessel (e.g. 25 ml per 125 ml Erlenmeyer shake flask).
2. Warm *Trans*IT-VirusGEN® GMP Reagent to room temperature and vortex gently.
3. Place 2.5 ml of PBS in a sterile tube.
4. In a separate sterile tube, combine the packaging plasmid premix (or individual plasmids) and transfer plasmid encoding the gene-of-interest. Mix thoroughly.
5. Transfer 25 µg of the total plasmid DNA prepared in Step B.4 to the tube containing PBS. Mix completely.
6. Add 75 µl of *Trans*IT-VirusGEN® GMP Reagent to the tube containing PBS and DNA. Mix completely by inversion or vortexing. Do NOT agitate Reagent:DNA complexes again after this initial mixing.
   NOTE: This is a 3:1 mixture of transfection reagent to total DNA (vol:wt), which can be further optimized for lentivirus production using *Trans*IT-VirusGEN® GMP Reagent.
7. Incubate the mixture at room temperature for 15-30 minutes stationary.
   NOTE: The ideal complex formation time may depend on the vector production platform but is typically between 5-60 minutes.

Do NOT allow the *Trans*IT-VirusGEN® GMP 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.

C. Distribute the complexes to cells in complete growth medium

1. Add the *Trans*IT-VirusGEN® GMP Reagent:DNA complexes (prepared in Step B) to the flask containing cells.
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 hours prior to lentivirus 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 lentivirus

1. Following the 48-hour incubation, centrifuge the lentivirus containing culture(s) in sterile conical tube(s) at 300 × g for 5 minutes. DO NOT dispose of supernatant following centrifugation.
2. Collect the virus-containing supernatant using a serological pipet into a sterile conical tube.
   NOTE: If a large batch of the same virus is produced, the supernatants can be combined.
3. Filter virus-containing supernatant through a 0.45 µm PVDF filter to remove any cells.
4. Immediately flash-freeze aliquots of lentivirus in cryogenic tubes and store at -80°C.

SECTION III: Lentivirus Transduction and Titering Protocol Using GFP Reporter Virus

The following procedure describes transduction of HEK 293T/17 cells grown in a 24-well format with a GFP reporter lentivirus to determine functional lentivirus titers. The number of wells needed for this assay will depend on the number of lentivirus stocks titered and the number of dilutions required for testing per stock (see Step B.5). Testing several dilutions is recommended to accurately determine the functional lentivirus titer.

Materials Required, but Not Supplied

- HEK 293T/17 cells (ATCC Cat. No. CRL-11268)
- Appropriate cell culture medium (e.g. DMEM + 10% FBS + 10 mM HEPES pH 7.4)
- Lentivirus stock(s) expressing GFP reporter
- *Transduce*IT™ Reagent (10 mg/ml, Mirus Cat. No. MIR 6620) or hexadimethrine bromide (Sigma Cat. No. H9268)
- 24-well tissue culture plate(s)
- 1X PBS and trypsin
- Flow cytometer equipped with a GFP compatible laser

A. Plate cells

1. Approximately 18-24 hours before transduction, plate HEK 293T/17 cells in 0.5 ml complete growth medium per well in a 24-well plate. A starting cell density of 2.0×10^5 cells/ml is recommended. Cultures should be ≥ 40% confluent at the time of transduction (see image at right).
   NOTE: Plate at least two extra wells to trypsinize and count on the day of transduction. An accurate cell count at the time of transduction is critical to determine an accurate functional titer (see B.1).
2. Incubate cell cultures at 37°C in 5% CO_2 overnight.

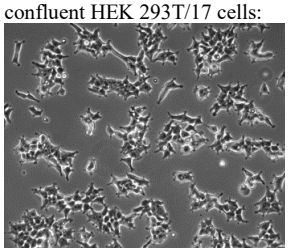
B. Transduce with GFP-encoding recombinant lentivirus

1. Trypsinize and count 2 wells of untransduced cells (plated in A.1) to obtain an accurate cell concentration at the time of transduction.
2. Dilute *Transduce*IT™ Reagent or hexadimethrine bromide to a working concentration of 16 µg/ml in pre-warmed complete growth medium (e.g. add 16 µl of a 10 mg/ml solution into 10 ml of growth medium).
3. Gently remove half of the medium from each well using a P1000 micropipettor.
4. Immediately add 250 µl of the *Transduce*IT™ or hexadimethrine bromide working solution to each well. The final concentration should be 8 µg/ml per well.
   NOTE: If transducing cell types other than HEK 293T/17, the optimal concentration of *Transduce*IT™ or hexadimethrine bromide should be empirically determined.
5. Add dilutions of the lentivirus stock to separate wells. Testing several dilutions is recommended to accurately determine functional titer. Guidelines are as follows:
   - For titers expected to be < 5.0×10^7 TU/ml, add 1 µl, 3 µl and 5 µl of the lentiviral stock to separate wells.
   - For titers expected to be ≥ 5.0×10^7 TU/ml, dilute the virus stock 10-fold in complete growth media. Add 1 µl, 3 µl and 5 µl of the diluted lentivirus stock to separate wells.

   NOTE: To obtain an accurate titer, it is desirable to have less than 20% GFP positive cells at 72 hours post-transduction. This minimizes counting cells with multiple integration events, which would result in an underestimation of titers.
6. Incubate the remaining assay wells at 37°C in 5% CO_2 for 72 hours post-transduction.

Passage cultured cells 18-24 hours before transduction to ensure active cell division at the time of transduction.

Representative image of ≥ 40% confluent HEK 293T/17 cells:



C. Cell harvest and analysis

1. Gently wash cells with 1X PBS and 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 to each well to inactivate the trypsin and resuspend the cells.
4. Transfer 100 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.
5. Add 150 µl of complete growth medium to each well to dilute the cells. This is required to obtain accurate flow cytometry results.
   NOTE: The optimal volume added for dilution may vary depending on the flow cytometer.
6. Analyze for GFP expression by flow cytometry.
7. Calculate the functional titer of the lentivirus stock using the following equation:

$$\text{Titer (Transducing Units/ml)} = \left[\frac{\text{Number of target cells (Count at time of transduction)} \times [\%\ \text{GFP positive cells}/100]}{\text{(Volume of lentivirus stock in ml)}} \right]$$

SECTION IV: AAV Generation in Adherent HEK 293 Cell Cultures

The following procedure describes plasmid DNA transfections for AAV generation in adherent HEK 293 cell types in a 6-well plate format. The surface areas of other culture vessels are different, and transfections must be scaled accordingly. Appropriately increase or decrease the amounts of serum free medium, *Trans*IT-VirusGEN® GMP Reagent, total plasmid DNA and complete culture medium based on the size of the cell culture vessel (refer to **Table 3** below).

Table 3. Recommended *Trans*IT-VirusGEN® GMP starting conditions for AAV production

Culture vessel	6-well plate	10-cm dish	T75 flask	T175 flask	Corning® 2-STACK	Corning® 5-STACK
Surface area	9.6 cm^2	59 cm^2	75 cm^2	175 cm^2	1272 cm^2	3180 cm^2
Complete growth medium	2 ml	10 ml	15 ml	35 ml	260 ml	650 ml
PBS or Serum-free medium	200 µl	1.0 ml	1.5 ml	3.5 ml	26 ml	65 ml
Total Plasmid DNA (1 µg/µl stock)	3 µl	15 µl	22.5 µl	52.5 µl	390 µl	975 µl
*Trans*IT-VirusGEN® GMP Reagent	6 µl	30 µl	45 µl	105 µl	780 µl	1.95 ml

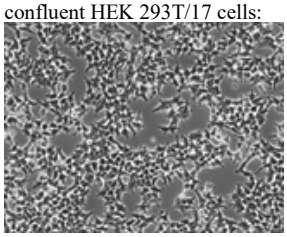
Surface areas are based on Falcon plates, dishes and flasks, and Corning CellSTACK® Culture Chambers. Volumes for cell growth and transfection complex formation are per culture vessel. For vessels not listed in this table, volumes of PBS, total DNA and *Trans*IT-VirusGEN® GMP Reagent can typically be scaled according to surface area (cm^2).

Materials Required but Not Supplied

- HEK 293 cells (e.g. HEK 293T/17 cells, ATCC Cat. No. CRL-11268)
- Complete culture medium (e.g. DMEM + 10% FBS + 10 mM HEPES pH 7.4)
- Plasmid DNA (e.g. pAAV-hrGFP (Agilent Cat. No. 240074-51), pHelper (Agilent Cat. No. 240071-54), pALD-AAV5 (Aldevron Cat. No. 5058-10))
- Phosphate Buffered Saline (PBS) (e.g. Millipore Sigma Cat. No. D8537)
- 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)

Passage cultured cells 18-24 hours before transfection to ensure active cell division at the time of transfection.

Representative image of ~80% confluent HEK 293T/17 cells:



Transient Plasmid Transfection Protocol per Well of a 6-Well Plate

A. Plate cells

1. Approximately 18-24 hours before transfection, plate cells in 2.0 ml complete growth medium per well in a 6-well plate. A starting cell density of 4.0 - 5.0 × 10^5 cells/ml is recommended. Cultures should be 80-95% confluent at the time of transfection (see representative image at right).
2. Incubate cell cultures at 37°C in 5% CO_2 overnight.

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

1. Warm *Trans*IT-VirusGEN® GMP to room temperature and vortex gently before using.
2. Place 200 µl of PBS in a sterile tube.
3. In a separate sterile tube, combine AAV plasmids per the manufacturer recommendations to a final concentration of 1 µg/µl. Mix thoroughly.
4. Transfer 3 µl of the prepared DNA mixture prepared in Step B.3 to the tube containing PBS. Mix completely.
5. Add 6 µl of *Trans*IT-VirusGEN® GMP Reagent to the diluted DNA mixture. Mix completely by inversion or vortexing. Do NOT agitate Reagent:DNA complexes again after this initial mixing.

Do NOT allow the *Trans*IT-VirusGEN® GMP 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.

NOTE: This is a 2:1 mixture of transfection reagent to total DNA (vol:wt), which can be further optimized for AAV production using *Trans*IT-VirusGEN® GMP Reagent.

6. Incubate the mixture at room temperature for 15-30 minutes stationary.
   NOTE: The ideal complex formation time may depend on the vector production platform but is typically between 5-60 minutes.

C. Distribute the complexes to cells in complete growth medium

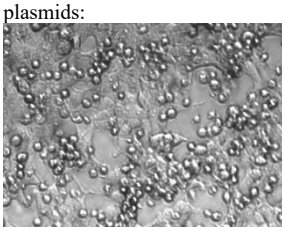
1. Add the *Trans*IT-VirusGEN® GMP Reagent:DNA complexes drop-wise to different areas of the wells.
2. Gently rock the culture vessel back-and-forth and from side-to-side to evenly distribute the *Trans*IT-VirusGEN® GMP Reagent:DNA complexes.
3. Incubate at 37°C in 5% CO_2 for 48-72 hours prior to AAV harvest.
   NOTE: It is not necessary to replace the complete growth medium with fresh medium post-transfection.

D. Harvest and storage of AAV

1. Following the 48-72 hour incubation, add 0.1X volume of 10X Cell Lysis Buffer (i.e. 0.2 ml) and 100 U/ml Benzonase® (i.e. 200 U) to each well. Incubate at 37°C for 1.5 hours with shaking.
2. Add 0.1X volume of 5 M NaCl (i.e. 0.2 ml) and mix completely. Incubate at 37°C for 30 minutes with shaking.
3. Transfer the entire cell lysate mixture to a sterile conical tube or appropriate vessel.
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.

Morphology changes in HEK 293T/17 cell following transfection with AAV plasmids are expected and indicate virus production.

Representative image of HEK 293T/17 cells 3 days post-transfection with pAAV-hrGFP, pAAV-RC and pHelper plasmids:



Benzonase® is a non-specific endonuclease used to liberate virus particles from residual nucleic acids in the cell lysates and increase AAV titers.

SECTION V: 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 serum-free complex medium, *Trans*IT-VirusGEN® GMP 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 4** (below).

Table 4. Scaling worksheet for *Trans*IT-VirusGEN® GMP Reagent for AAV transfections

Starting conditions per milliliter of complete growth medium (AAV Generation)					
	Per 1 ml		Total culture volume		Reagent quantities
PBS or Serum-free Medium	0.1 ml	×	_____ml	=	_____ml
Total Plasmid DNA (1 µg/µl stock)	2 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 (Irivine Scientific Cat. No. 91165))
- Plasmid DNA (e.g. pAAV-hrGFP (Agilent Cat. No. 240074-51), pHelper (Agilent Cat. No. 240071-54), pALD-AAV5 (Aldevron Cat. No. 5058-10))
- 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)

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).

Passage 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 - 3 × 10^6 cells/ml into a transfection culture vessel (e.g. 25 ml per 125 ml Erlenmeyer shake flask).
2. Warm *Trans*IT-VirusGEN® GMP to room temperature and vortex gently before using.
3. Place 2.5 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 50 µl of the DNA mixture prepared in Step B.4 to the tube containing PBS. Mix completely.
6. Add 75 µl of *Trans*IT-VirusGEN® GMP Reagent to the diluted DNA. 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 the mixture at room temperature for 15-30 minutes stationary.
   NOTE: The ideal complex formation time may depend on the vector production platform but is typically between 5-60 minutes.

Do NOT allow the *Trans*IT-VirusGEN® GMP 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.

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.
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.

SECTION VI: 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.

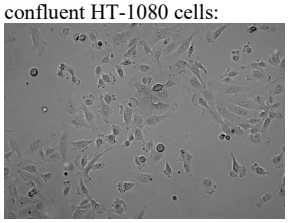
   NOTE: To obtain an accurate titer, it is desirable to have less than 20% GFP positive cells at 48 hours post-transduction. This minimizes counting cells with multiple integration events, which would result in an underestimation of titer.

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 HEK 293 cultures and harvested as described in Section IV.D and V.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® GMP Reagent:DNA ratio for each cell type. Titrate the *Trans*IT-VirusGEN® GMP Reagent volume from 2-4 µl (lentivirus) or 1-3 µl (AAV) 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 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. For lentivirus, start with 1 µg DNA per 1 ml of culture. For AAV, 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. Remove 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.
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 viral vector harvest not optimal	Determine the optimal time post-transfection to harvest viral vectors. Typically, the best time is 48 hours post-transfection to harvest lentivirus and 48-72 hours post-transfection to harvest AAV. However, 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 mix gently before each use. If *Trans*IT-VirusGEN® GMP Reagent is pre-diluted in complex formation solution, DNA should be added within 5 min. 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 and *Trans*IT-VirusGEN® GMP Reagent, do not agitate Reagent:DNA complexes again, e.g. do not vortex or invert before adding to cultures.
Excessive complex formation time	We recommend a complex formation time of 15-30 minutes, though viral titer and quality may be further optimized by evaluating complex formation times between 5-60 minutes for each unique vector construct.
Precipitate formation or turbid appearance 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® GMP 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. 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 16). 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.

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 less than 80% confluent at the time of transfection. For high virus titers using *Trans*IT-VirusGEN® GMP Reagent, ensure that cell cultures are between 80 and 95% confluent (for adherent cell transfections) or approximately 2 - 3 × 10^6 cells/ml (for suspension cell transfections) at the time of transfection.
Cell morphology has changed	When generating lentivirus, overexpression of the vesicular stomatitis virus (VSV) G protein causes changes in cell morphology and can even result in cell-cell fusion. 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 drop-wise to the cells. For adherent cell cultures, gently rock the dish back-and-forth and from side-to-side (instead of rotating) to distribute the complexes evenly. For suspension cultures, 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. Avoid vigorous agitation that could disturb formed transfection complexes, e.g. vortexing after initial mixing of the DNA and transfection reagent.
Transfection complexes added to adherent cells cultured in serum-free medium	*Trans*IT-VirusGEN® GMP Transfection Reagent efficiently transfects cells cultured in serum-free medium; however, toxicity may be higher if serum is not present when transfecting adherent cells typically cultured in serum-containing complete media. If toxicity is a problem, consider adding serum to the culture medium.

RELATED PRODUCTS

- VirusGEN® GMP LV Transfection Kit
- VirusGEN® LV Transfection Kit
- VirusGEN® GMP AAV Transfection Kit
- VirusGEN® AAV Transfection Kit
- *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

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