VirusGEN® LV Transfection Kit

Protocol for MIR 6760, 6765, 6792

Quick Reference Protocol, SDS and Certificate of Analysis available at mirusbio.com

INTRODUCTION

Lentivirus is an enveloped, single-stranded RNA virus from the *Retroviridae* family capable of infecting both dividing and non-dividing cells. Combined with an efficient host-genome integration mechanism and the ability to pseudotype the virus, this capability makes recombinant lentivirus a central gene delivery tool for robust and stable transgene expression in target cells.

The *Trans*IT-VirusGEN® Transfection Reagent enables the generation of high titer lentivirus in HEK 293 cell types. The VirusGEN® LV Transfection Kit further enhances the performance of *Trans*IT-VirusGEN® Transfection Reagent in adherent and suspension HEK 293 cells through inclusion of the proprietary VirusGEN® LV Complex Formation Solution and VirusGEN® LV Enhancer. The *Trans*IT-VirusGEN® LV Transfection Kit is ideal for generating high titer lentivirus preparations to accelerate research and development.

SPECIFICATIONS

Storage	Store *Trans*IT-VirusGEN® Reagent at -10 to -30°C, tightly capped. Store VirusGEN® LV Enhancer at 2 to 10°C. Store VirusGEN® LV Complex Formation Solution at 2 to 10°C. ***Before each use***, warm to room temperature and mix gently.
Stability / Guarantee	When properly stored and handled, *Trans*IT-VirusGEN® Transfection Reagent is guaranteed for 1 year from the date of purchase, and VirusGEN® LV Complex Formation Solution and VirusGEN® LV Enhancer are guaranteed for 6 months from the date of purchase.

Warm *Trans*IT-VirusGEN® Reagent, VirusGEN® LV Complex Formation Solution and VirusGEN® LV Enhancer to room temperature before each use. Mix gently.

MATERIALS

Materials Supplied

The VirusGEN® LV Transfection Kit is supplied in ***one*** of the following formats.

Product No.	Volume of *Trans*IT-VirusGEN® Transfection Reagent	Volume of VirusGEN® LV Complex Formation Solution	Volume of VirusGEN® LV Enhancer
MIR 6760	2 × 1.5 ml	1 × 100 ml	1 × 100 ml
MIR 6765	1 × 30 ml	1 × 1 L	1 × 1 L
MIR 6792	1 × 150 ml	5 × 1 L	5 × 1 L

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

For Research Use Only

BEFORE YOU START:

Important Tips for Optimal Lentivirus Production

The suggestions below yield high efficiency plasmid DNA transfection using the VirusGEN® LV Transfection Kit.

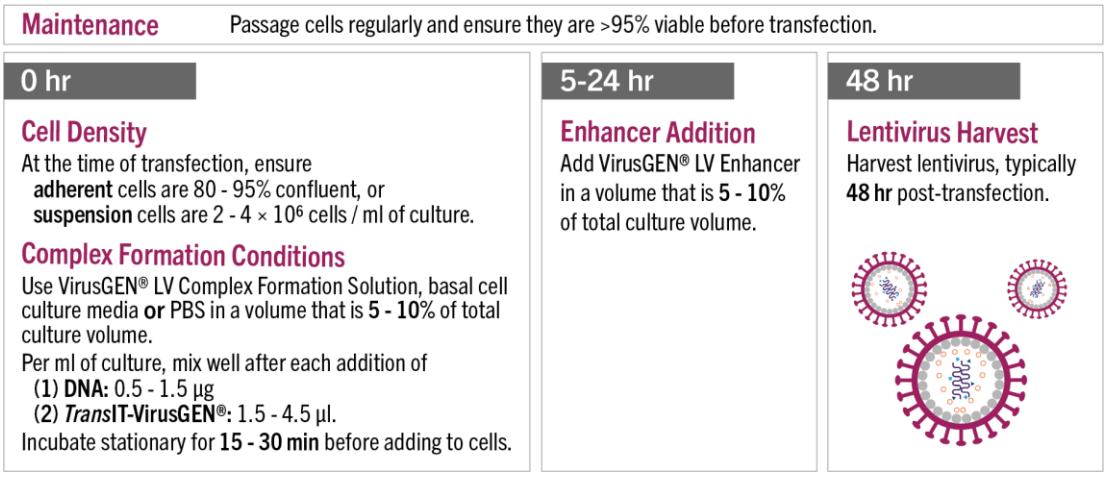
- **Cell culture conditions.** Culture cells in the appropriate medium, with or without serum (e.g. DMEM + 10% FBS + 10 mM HEPES pH 7.4 for adherent HEK 293 cultures; BalanCD HEK293 for suspension HEK 293 cultures). Ensure cells are ≥ 95% viable by trypan blue exclusion and doubling every 24 hours. After 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 - 4 × 10^6 cells/ml. Passage cells 18-24 hours before transfection to ensure that cells are actively dividing and reach the appropriate density at transfection.
- **Lentivirus 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® to DNA.** Determine the optimal *Trans*IT-VirusGEN® Reagent:DNA ratio for each cell type by varying the amount of reagent from 2-4 µl per 1 µg total DNA. Refer to **Tables 1** and **2** for recommended starting conditions based on culture size.
- **Complex formation conditions.** Prepare *Trans*IT-VirusGEN® Reagent:DNA complexes in VirusGEN® LV Complex Formation Solution, 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.
- **VirusGEN® LV Enhancer Addition.** We recommend adding the VirusGEN® LV Enhancer at a volume that is 5-10% of the total culture volume, approximately 5-24 hours post-transfection. Addition at an earlier time may result in lower titer. Mirus recommends comparing transfection +/- Enhancer because, with some cell lines and vector constructs, equivalently high titer can be obtained *without* the Enhancer component.

Premix packaging and transfer plasmids together prior to adding to the complex formation medium.

Do not use serum or antibiotics in the media during transfection complex formation.

Transfection complexes can be added directly to cells cultured in growth media +/- serum and up to 0.1-1X antibiotics.

VirusGEN® LV Transfection Kit Workflow:



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 VirusGEN® LV Complex Formation Solution, *Trans*IT-VirusGEN® Reagent, total plasmid DNA, complete culture medium and VirusGEN® LV Enhancer based on the size of the cell culture vessel (refer to **Table 1** below).

Table 1. Recommended starting conditions for VirusGEN® LV Transfection Kit

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	2.0 ml	10 ml	15 ml	35 ml	260 ml	650 ml
VirusGEN® LV Complex Formation Solution	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® Reagent	6 µl	30 µl	45 µl	105 µl	780 µl	1.95 ml
NOTE: Add VirusGEN® LV Enhancer 5-24 hours post-transfection.						
VirusGEN® LV Enhancer	200 µl	1.0 ml	1.5 ml	3.5 ml	26 ml	65 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® Reagent can typically be scaled according to surface area (cm^2).

Mirus recommends comparing transfection +/- Enhancer because, with some cell lines and vector constructs, equivalently high titer can be obtained *without* the Enhancer component.

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

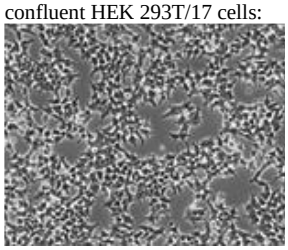
Transient Plasmid Transfection Protocol per Well of a 6-Well Plate

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 - 6 × 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.

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:



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

1. Warm *Trans*IT-VirusGEN® Transfection Reagent to room temperature and vortex gently before using.
2. Place 200 µl of VirusGEN® LV Complex Formation Solution 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 VirusGEN® LV Complex Formation Solution. Mix completely.
5. Add 6 µl of *Trans*IT-VirusGEN® 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, which can be further optimized for lentivirus production using *Trans*IT-VirusGEN® 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® 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® Reagent:DNA complexes.
3. Incubate cultures for 5-24 hours before addition of VirusGEN® LV Enhancer.

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. Add VirusGEN® LV Enhancer to transfected cell culture

1. After 5-24 hours post-transfection, add 200 µl of VirusGEN® LV Enhancer to the culture vessel containing the transfected cell culture. Gently rock the culture vessel to mix completely.
   NOTE: Mirus recommends comparing transfection +/- Enhancer because, with some cell lines and vector constructs, equivalently high titer can be obtained *without* the Enhancer component.
2. Incubate cultures for an additional 24-43 hours, i.e. 48 hours post-transfection, before harvesting lentivirus.

E. 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 VirusGEN® LV Complex Formation Solution, *Trans*IT-VirusGEN® Reagent, total plasmid DNA and VirusGEN® LV Enhancer 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).

Table 2. Scaling worksheet for VirusGEN® LV Transfection Kit

Starting conditions per milliliter of complete growth medium						
	Per 1 ml			Total culture volume		Reagent quantities
VirusGEN® LV Complex Formation Solution	0.1	ml	×	_____ml	=	_____ml
Total plasmid DNA (1 µg/µl stock)	1	µl	×	_____ml	=	_____µl
*Trans*IT-VirusGEN® Reagent	3	µl	×	_____ml	=	_____µl
NOTE: Add VirusGEN® LV Enhancer 5-24 hours post-transfection.						
	Per 1 ml			Total culture volume		Reagent quantities
VirusGEN® LV Enhancer	0.1	ml	×	_____ml	=	_____ml

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.

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 4 - 6 × 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 - 4 × 10^6 cells/ml into a transfection culture vessel (e.g. 25 ml per 125 ml Erlenmeyer shake flask).
2. Warm *Trans*IT-VirusGEN® Reagent to room temperature and vortex gently.
3. Place 2.5 ml of VirusGEN® LV Complex Formation Solution 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 (GOI). Mix thoroughly.
5. Transfer 25 µg of the total plasmid DNA prepared in Step B.4 to the tube containing VirusGEN® LV Complex Formation Solution. Mix completely.
6. Add 75 µl *Trans*IT-VirusGEN® 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, which can be further optimized for lentivirus production using *Trans*IT-VirusGEN® 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® 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® Reagent:DNA complexes (prepared in Step B) to the flask containing cells. Swirl the flask gently to mix completely.
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 5-24 hours before addition of VirusGEN® LV Enhancer.

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

D. Add VirusGEN® LV Enhancer to transfected cell culture

1. After 5-24 hours post-transfection, add 2.5 ml of VirusGEN® LV Enhancer to the culture vessel containing the transfected cell culture. Swirl the flask gently to mix completely.
2. Continue shaking the 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 an additional 24-43 hours, i.e. 48 hours post-transfection, before harvesting lentivirus.

E. 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 being produced, the supernatants can be combined.
3. Filter the virus-containing supernatant through a 0.45 µm PVDF filter (e.g. Millipore Steriflip-HV, Cat. No. SE1M003M00) 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

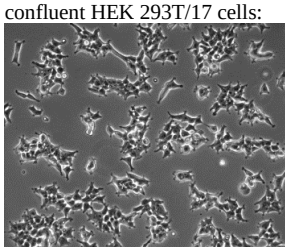
- HEK 293T/17 cells (ATCC Cat. No. CRL-11268)
- Appropriate cell culture medium (e.g. DMEM + 10% FBS + 10 mM HEPES pH 7.4)
- Lentivirus stock(s) expressing GFP reporter
- *Transduce*IT™ Reagent (10 mg/ml, Mirus Cat. No. MIR 6620) or hexadimethrine bromide (Sigma Cat. No. H9268)
- 24-well tissue culture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

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:



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.

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:

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

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:DNA complex formation conditions	Determine the best *Trans*IT-VirusGEN® Reagent:DNA ratio for each cell type. Titrate the *Trans*IT-VirusGEN® Reagent volume from 2-4 µl per 1 µg DNA. Refer to "Before You Start" on Page 2 for recommended starting conditions.
	Compare transfection +/- Enhancer because, with some cell lines and vector constructs, equivalently high titer can be obtained *without* the Enhancer component.
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 0.5-1.5 µg per 1 ml of culture. Start with 1 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 Mirus MiraCLEAN® Endotoxin Removal Kit (MIR 5900) for removal of 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 lentivirus harvest not optimal	Determine the optimal transfection incubation time for each cell type and experiment. Test a range of incubation times (e.g. 48-72 hours). The best post-transfection incubation time for lentivirus production is typically 48 hours.
*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 min. Incubating the *Trans*IT-VirusGEN® 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® 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 tables in the protocol, including serum-free media, *Trans*IT-VirusGEN® 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.
	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 11).
	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 less than 80% confluent at the time of transfection. For high virus titers using *Trans*IT-VirusGEN® Reagent, ensure that cell cultures are between 80 and 95% confluent (for adherent cell transfections) or approximately 2 - 4 × 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. VirusGEN® LV Enhancer may also decrease cell growth. 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® 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® GMP AAV Transfection Kit
- VirusGEN® AAV Transfection Kit
- *Trans*IT-VirusGEN® GMP Transfection Reagent
- *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

Reagent Agent®

Reagent Agent® is an online tool designed to help determine the best solution for nucleic acid delivery based on in-house data, customer feedback and citations.

Learn more at:
www.mirusbio.com/ra

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