VirusGEN™ LV Production System

Alpha Test Protocol for LV Generation in Suspension 293 Cells

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 VirusGEN™ LV Production System harnesses the performance of the *Trans*IT-VirusGEN® Transfection Reagent, which was developed by screening lipid and polymer libraries to identify a reagent formulation that enhances delivery of packaging and transfer vectors to HEK 293 cells, and a proprietary LV Enhancer solution that increases lentivirus titers multi-fold over existing LV production systems. With salient features including: high efficiency DNA delivery, culture format versatility and scalability, and streamlined virus generation workflows, the VirusGEN™ LV Production System and is ideal for scientists generating large-scale lentivirus preparations.

SPECIFICATIONS

Storage	Store *Trans*IT-VirusGEN® Transfection Reagent tightly capped at –20°C. ***Before each use***, warm to room temperature and vortex gently. Store the VirusGEN™ LV Complex Formation Solution at 4°C. Store the VirusGEN™ LV Enhancer at 4°C.
Product Guarantee	*Trans*IT-VirusGEN® Transfection Reagent is guaranteed for 6 months from date of purchase, when properly stored and handled. Expiry information is not yet available for VirusGEN™ LV Complex Formation Solution and VirusGEN™ LV Enhancer.

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

MATERIALS

Materials Supplied

The VirusGEN™ LV Production System is supplied in the following format. For bulk quantities of the kit or individual components, please inquire about a custom quote.

Product No.	Component	Volume
MIR 6700	*Trans*IT-VirusGEN® Transfection Reagent	2 x 1.5 ml
MIR XXXX	VirusGEN™ LV Complex Formation Solution	1 x 100 ml
MIR XXXX	VirusGEN™ LV Enhancer	1 x 100 ml

For Materials Required but Not Supplied, see Protocol Sections:

- (I) Lentivirus Generation in Suspension HEK 293-F Cell Cultures
- (II) Lentivirus Transduction and Titering Protocol Using GFP Reporter Virus

For Research Use Only

BEFORE YOU START:

Important Tips for Optimal Lentivirus Production

Mirus recommends using FreeStyle™ 293-F Cells (Life Technologies® Cat. No. R790-07) for high titer lentivirus production in suspension HEK 293 cultures. The suggestions below yield high efficiency plasmid DNA transfection using the VirusGEN™ LV Production System.

- **Cell density at transfection.** The recommended cell density for suspension 293-F cells is 4 x 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.
- **DNA purity.** Use highly purified, sterile, endotoxin-free and contaminant-free DNA for transfection. Plasmid DNA preparations that have an $A_{260/280}$ absorbance ratio of 1.8–2.0 are desirable. DNA prepared using miniprep kits is not recommended as it might contain high levels of endotoxin. We recommend using MiraCLEAN® Endotoxin Removal Kit (MIR 5900) to remove endotoxin from your DNA preparation.
- **Lentivirus packaging and transfer plasmids.** The *Trans*IT-VirusGEN® Reagent was optimized using a lentivirus packaging vector pre-mix. If using individual packaging plasmids, we recommend a starting ratio of 4 µg *gag-pol* vector, 1 µg *rev* vector and 1 µg VSV-G vector. Premix the packaging plasmids with an equal amount of the transfer vector (e.g. 6 µg) to maintain a 1:1 (wt:wt) ratio of packaging to transfer plasmids.
- **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 *Trans*IT-VirusGEN® Reagent from 2-4 µl per 1 µg total DNA. Refer to **Tables 1** for recommended starting conditions based on cell culture vessel size.
- **Complex formation conditions.** Prepare *Trans*IT-VirusGEN® Reagent:DNA complexes in VirusGEN™ LV Complex Formation Solution (NOTE: We typically recommend forming transfection complexes in 10% of total culture volume. It is possible to form more concentrated transfection complexes with the VirusGEN™ LV Complex Formation Solution if desired. Please keep in mind that transfection complexes form more rapidly in lower volumes of complex formation solution).
- **Cell culture conditions.** Culture cells in the appropriate complete growth medium (e.g. FreeStyle™ F17 + 4mM L-Glutamine + 0.2% Poloxamer 188 for suspension 293 culture). Not all serum free complete growth media support transient transfection. After transfection, there is no need to perform a medium change to remove the transfection complexes.
- **VirusGEN™ LV Enhancer addition.** We recommend adding the VirusGEN™ LV Enhancer at 10% total culture volume, approximately 18-24 hours post-transfection (e.g. Add 100 ml VirusGEN™ LV Enhancer per 1L total transfected culture). We do NOT recommend adding enhancer earlier than 18 hours post-transfection as this may lead to lower LV titers.
- **Presence of antibiotics.** Antibiotics inhibit transfection complex formation and should be excluded from the complex formation step. Transfection complexes can be added directly to cells growing in complete culture medium containing serum and low levels of antibiotics (0.1–1X final concentration of penicillin/streptomycin mixture).
- **Media change post-transfection.** A media change is not required and could be detrimental to virus titers; therefore, we do not recommend a media change post-transfection.
- **Post-transfection incubation time for lentivirus.** The optimal incubation time for harvesting high titer lentivirus is 48 hours. Minimal amounts of functional lentivirus are produced during the period of 48-72 hours post-transfection.

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 Suspension 293F 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, 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 1** for transfection and **Table 2 for t**he VirusGEN™ LV Enhancer.

Table 1. Calculation worksheet for scaling transfections with the VirusGEN™ System

Starting conditions per milliliter of complete growth medium						
	Per 1 ml		Total culture volume		Reagent quantities	
VirusGEN™ LV Complex Formation Solutio	0.1 ml	×	_____ml	=	_____ml	
Transfer plasmid DNA (1µg/µl stock)	0.5 µl	×	_____ml	=	_____µl	
Packaging DNA premix (1µg/µl stock)	0.5 µl	×	_____ml	=	_____µl	
*Trans*IT-VirusGEN® Transfection Reagent	3 µl	×	_____ml	=	_____µl	

Table 2. Calculation worksheet for VirusGEN™ LV Enhancer scaling

Starting conditions per milliliter of complete growth medium **NOTE: Add enhancer to culture 18-24 hours post-transfection.**					
	Per 1 ml	Total culture volume		Reagent quantities	
VirusGEN™ LV Enhancer	0.1 ml	×	_____ml	=	_____ml

Materials Required but Not Supplied

- FreeStyle™ 293-F cells (Life Technologies® Cat. No. R790-07) or equivalent
- Complete Culture Medium (e.g. FreeStyle™ F17 + 4mM L-Glutamine + 0.2% Poloxamer 188)
- Nucleic acid (2nd or 3rd generation packaging plasmids and transfer vector with GOI)
- Erlenmeyer shake flasks (e.g. Corning® Cat. No. 431143 or Thomson Cat. No. 931110)
- 50 ml conical tube(s) for virus collection
- 0.45 µm PVDF filter (e.g. Millipore Cat. No. SE1M003M00 or SLHV033RS)
- Reporter assay as required

Transient Plasmid Transfection Protocol per 25 ml 293-F Culture

A. Maintenance of Cells

1. Passage suspension 293-F 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 x 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).

Divide 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 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. Add 25 µg total plasmid DNA (i.e. 12.5 µg Transfer vector encoding GOI + 8.3 µg Gag-pol vector + 2.1 µg Rev vector + 2.1 µg VSV-G vector). Pipet gently to mix completely.
5. Add 75 µl *Trans*IT-VirusGEN® Transfection Reagent to the diluted DNA. Vortex gently to mix completely.
6. Incubate at room temperature for 10-15 minutes to allow transfection complexes to form.

*Trans*IT-VirusGEN® Reagent was optimized using a pre-mix of lentivirus packaging vectors. If using individual packaging plasmids, we recommend a starting ratio of 4 µg *gag-pol* vector, 1 µg *rev* vector and 1 µg VSV-G vector. Premix the packaging plasmids with an equal amount of the transfer vector (e.g. 6 µg) to maintain a 1:1 (wt:wt) ratio of packaging to transfer plasmids.

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 18-24 hours, then proceed to step D below (Enhancer addition).

D. Add VirusGEN™ LV Enhancer to transfection culture

1. Between 18-24 hours post transfection, add 2.5 mL of VirusGEN™ LV Enhancer Solution into the transfection culture vessel containing 25 ml of transfected 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 24-30 hours prior to virus harvest (total of 48 hours post-transfection).

E. Virus Harvest

1. Following the 48-hour incubation, centrifuge the culture in sterile conical tube at 300 x 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 II: 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.4). Testing several dilutions is recommended to accurately determine the functional lentivirus titer.

Materials Required, but not Supplied

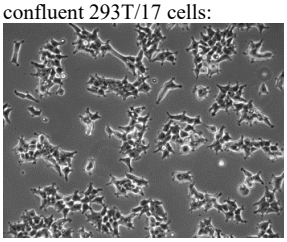
- HEK 293T/17 cells (ATCC Cat. No. CRL-11268)
- Appropriate cell culture medium (e.g. DMEM + 10% FBS + 10mM HEPES pH 7.4)
- Lentivirus stock(s) expressing GFP reporter
- *Transduce*IT™ Reagent (10 mg/ml, Mirus Cat. No. MIR 6620) or hexadimethrine bromide (Sigma, Cat. No. H9268)
- 24-well tissue culture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

Divide cultured cells 18–24 hours before transfection to ensure active cell division at the time of transduction.

Representative image of ≥ 40% confluent 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 1µl, 3µl and 5µl 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 1µl, 3µl and 5µl 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 the number of cells transduced by 2 different viruses.
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 rounded, 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:

$$\text{Titer (Transducing units/ml)} = \left[\frac{\text{Number of target cells (Count at day 2, transduction) x [\% GFP positive cells/100]}}{\text{(Volume of lentivirus stock in ml)}} \right]$$

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 the transfer vector plasmid DNA.
Suboptimal *Trans*IT® Reagent:DNA ratio	Determine the best *Trans*IT-VirusGEN® Reagent:DNA ratio for each cell type. Titrate the *Trans*IT-VirusGEN® Reagent volume from 1.5–4 µl (lentivirus) per 1 µg DNA. Refer to "Before You Start" on Page 2.
Suboptimal DNA concentration	Determine the DNA concentration accurately. Use plasmid DNA preps that have an $A_{260/280}$ absorbance ratio of 1.8–2.0. The optimal DNA concentration generally ranges between 1–3 µg/well of a 6-well plate. Start with 2.0 µg/well of a 6-well plate. Consider testing more or less 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. **Do not** use DNA prepared using miniprep kits as it might contain high levels of endotoxin.
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.
Transfection incubation time	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 48 hours; the best post-transfection incubation time for AAV is 72 hours.
*Trans*IT-VirusGEN® was not mixed properly	Warm *Trans*IT-VirusGEN® Reagent to room temperature and vortex gently before each use.
Precipitate formation 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® 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.
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 15). 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.

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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 approximately 4 x 10^6 cells/ml (suspension) 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 293T/17 cells below passage 30 for optimal recombinant lentivirus production
Transfection complexes not evenly distributed after complex addition to cells	Add transfection complexes drop-wise to the cells. Gently rock the dish back-and-forth and from side-to-side to distribute the complexes evenly. Do not swirl or rotate the dish, as this may cause uneven distribution.
Transfection complexes added to 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. If toxicity is a problem, consider adding serum to the culture medium.

RELATED PRODUCTS

- Ingenio® Electroporation Solution and Kits
- *Label*IT® Plasmid Delivery Controls
- *Label*IT® RNAi Delivery Controls
- *Label*IT® Tracker™ Intracellular Nucleic Acid Localization Kits
- MiraCLEAN® Endotoxin Removal Kits
- *Trans*IT®-Lenti Transfection Reagent
- *Trans*IT-X2® Dynamic Delivery System
- *Trans*IT®-2020 Transfection Reagent
- *Trans*IT®-LT1 Transfection Reagent
- *Transduce*IT™ Reagent

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.

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