

TransIT® Lentivirus System

Protocol for MIR 6650, 6655



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

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, this capability has led to recombinant lentivirus becoming a central gene delivery tool for robust and stable transgene expression in target cells. However, the lentivirus platform is often limited by insufficient viral titers requiring concentration before use. To address this limitation, Mirus Bio developed the *TransIT*® Lentivirus System for enhanced delivery and expression of the essential *gag*, *pol*, *rev* and VSV-G genes required for higher-titer lentivirus production in adherent or suspension HEK 293 derived cell types.

The *TransIT*® Lentivirus System is composed of *TransIT*®-Lenti Transfection Reagent, Lentivirus Packaging Mix Powered by MISSION® Genomics, and *TransducelIT*™ Reagent for enhanced lentiviral transductions.

SPECIFICATIONS

Storage	Store <i>TransIT</i> ®-Lenti Transfection Reagent, Lentivirus Packaging Mix, and <i>TransducelIT</i> ™ Reagent tightly capped at –20°C. Before each use , warm to room temperature and vortex gently.
Product Guarantee	<i>TransIT</i> ®-Lenti Transfection Reagent, Lentivirus Packaging Mix Powered by MISSION® Genomics and <i>TransducelIT</i> ™ Reagent are guaranteed for 1 year from date of purchase, when properly stored and handled.

MATERIALS

TransIT® Lentivirus System is supplied in the following configurations. For bulk quantities of the kit or individual components, please inquire about a custom quote.

<i>TransIT</i> ® Lentivirus System	Component Product No.	Component	Quantity
MIR 6650	MIR 6603	<i>TransIT</i> ®-Lenti Transfection Reagent	0.3 mL
	MIR 6630	Lentivirus Packaging Mix*	5 rxn (0.25 mL)
	MIR 6620	<i>TransducelIT</i> ™ Reagent	1 mL
MIR 6655	MIR 6600	<i>TransIT</i> ®-Lenti Transfection Reagent	1.5 mL
	MIR 6640	Lentivirus Packaging Mix*	34 rxn (1.7 mL)
	MIR 6620	<i>TransducelIT</i> ™ Reagent	1 mL



The number of reactions per kit is determined by the amount of *TransIT*®-Lenti Reagent and Lentivirus Packaging Mix required for one 10-cm dish transfection.

*Lentivirus Packaging Mix Powered by MISSION® Genomics. MISSION® is a registered trademark of Sigma-Aldrich® Co. LLC.

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BEFORE YOU START:

Important Tips for Optimal Lentivirus Production

Mirus recommends using HEK 293T/17 cells or FreeStyle[™] 293-F Cells for high titer lentivirus production. The suggestions below yield high efficiency plasmid DNA transfection using the TransIT[®] Lentivirus System.

- **Cell density (% confluence) at transfection.** The recommended cell density for:
 - Adherent HEK 293T/17 cells is 80 - 95% confluence. DO NOT transfect cells at < 80% confluency as this may result in high cytotoxicity and low virus titers.
 - Suspension HEK 293-F cells is 2×10^6 cells/mL. Passage cells 18–24 hours before transfection to ensure that cells are actively dividing (i.e. doubling every 24 hours) at the time of transfection. DO NOT transfect if cells are < 95% viable at time of transfection.
- **Suspension cell maintenance.** Maintain HEK 293-F cultures between 5×10^5 and 6×10^6 cells/mL. Perform cell counts and viability daily to ensure that the cells are doubling every 24 hours and > 95% viability.
- **DNA purity.** Use highly pure, sterile, and endotoxin-free DNA for transfection with a $A_{260/280}$ absorbance ratio of 1.8–2.0. 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.
- **Packaging and transfer plasmids.** The Lentivirus Packaging Mix Powered by MISSION[®] Genomics is an optimized mixture of two plasmids expressing the *gag*, *pol*, *rev* and VSV-G genes required for higher-titer lentivirus production. For transfection, premix Lentivirus Packaging Mix with an equal amount of 2nd or 3rd generation lentivirus transfer vector containing the gene of interest (GOI) to maintain a 1:1 (wt:wt) ratio of packaging to transfer plasmids.
- **Ratio of TransIT[®]-Lenti to DNA.** Determine the best TransIT[®]-Lenti Reagent:DNA ratio for each cell type. Vary the amount of TransIT[®]-Lenti Reagent from 2–4 μ L per 1 μ g DNA to find the optimal ratio. Refer to **Tables 1 and 2** for recommended starting conditions based on cell culture vessel size.
- **Complex formation conditions.** Prepare TransIT[®]-Lenti Reagent:DNA complexes in serum-free growth medium (e.g. Opti-MEM[®] I Reduced-Serum Medium).
- **Presence of antibiotics.** Antibiotics should not be in the serum-free media used to mix DNA and transfection reagent. Antibiotics can be present in the complete media to grow cells.
- **Cell culture conditions.** Culture HEK 293 cells in the appropriate medium, with or without serum (e.g. DMEM + 10% FBS + 10mM HEPES pH 7.4 for adherent HEK 293T cultures; FreeStyle[™] F17 + 4mM L-Glutamine + 0.2% Poloxamer 188 for suspension HEK 293 cultures).
- **Media change post-transfection.** A media change is not required and could be detrimental to lentivirus titers.
- **Post-transfection incubation time.** The optimal incubation time for harvesting high titer lentivirus is 48 hours.



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



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

SECTION I: Lentivirus Generation in Adherent HEK 293T Cell Cultures

The following procedure describes plasmid DNA transfections for lentivirus generation in adherent HEK 293T cell types in a 6-well plate format. If using an alternative culture vessel, increase or decrease the amounts of serum-free medium, TransIT®-Lenti 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 TransIT® Lentivirus System starting conditions

Culture vessel	48-well plate	24-well plate	12-well plate	6-well plate	10-cm dish	T75 flask
Surface area	1.0 cm ²	1.9 cm ²	3.8 cm ²	9.6 cm ²	59 cm ²	75 cm ²
Complete growth medium	263 µL	0.5 mL	1.0 mL	2.0 mL	10 mL	15 mL
Serum-free medium	26 µL	50 µL	100 µL	200 µL	1.0 mL	1.5 mL
Transfer DNA (1 µg/µL stock)	0.13 µL	0.25 µL	0.5 µL	1.0 µL	5 µL	7.5 µL
Lentivirus Packaging Mix* (0.1 µg/µL stock)	1.3 µL	2.5 µL	5.0 µL	10 µL	50 µL	75 µL
TransIT®-Lenti Reagent	0.78 µL	1.5 µL	3 µL	6 µL	30 µL	45 µL

*Lentivirus Packaging Mix Powered by MISSION® Genomics. MISSION® is a registered trademark of Sigma-Aldrich® Co. LLC.



Surface areas are based on Greiner tissue culture plates, Falcon 10-cm dishes and T75 flasks. Volumes are per well (or dish) for a given culture vessel.

If small volumes of TransIT®-Lenti need to be pipetted, dilute the reagent in serum-free medium before each use to avoid pipetting errors. **Do not** store diluted TransIT®-Lenti Reagent.

Materials Required but Not Supplied

- HEK 293T cells (e.g. HEK 293T/17 cells, ATCC Cat. No. CRL-11268)
- Complete culture medium (e.g. DMEM + 10% FBS + 10mM HEPES pH 7.4)
- Nucleic Acid (i.e. 2nd or 3rd generation transfer plasmid containing GOI)
- Serum-free medium (e.g. Opti-MEM® I Reduced-Serum Medium)
- 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 4.0 – 5.0 × 10⁵ cells/mL is recommended. Cultures should be 80 - 95% confluent at the time of transfection. DO NOT transfect cells at a lower confluency as this may lead to high cytotoxicity and lower lentivirus titers.
2. Incubate cell cultures at 37°C in 5% CO₂ overnight.

B. Prepare TransIT®-Lenti:DNA complexes (Immediately before transfection)

1. Warm TransIT®-Lenti Reagent to room temperature and vortex gently before using.
2. Place 200 µL of Opti-MEM® I Reduced-Serum Medium in a sterile tube.
3. In a separate sterile tube, combine 10 µL (1 µg) Lentivirus Packaging Mix and 1 µg transfer plasmid DNA encoding the transgene of interest. Mix thoroughly.
4. Transfer 2.0 µg of the DNA prepared in Step B.3 (1 µg Lentivirus Packaging Mix + 1 µg transfer plasmid) to the tube containing Opti-MEM® I Reduced-Serum Medium.
5. Pipet gently to mix completely.
6. Add 6.0 µL TransIT®-Lenti Reagent to the diluted DNA mixture.
7. Pipet gently to mix completely.
8. Incubate at room temperature for 10 minutes to allow transfection complexes to form.

C. Distribute the complexes to cells in complete growth medium

1. Add the *TransIT*[®]-Lenti 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 *TransIT*[®]-Lenti Reagent:DNA complexes.
3. Incubate at 37°C in 5% CO₂ for 48 hours.

NOTE: It is not necessary to replace the complete growth medium with fresh medium post-transfection.

D. Harvest and storage of lentivirus

1. Harvest cell supernatant containing recombinant lentivirus particles.
NOTE: If cells detach during harvest, centrifuge cells at 300 x 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-F 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 an alternate cell culture vessel, increase or decrease the amounts of serum-free complex medium, *TransIT*[®]-Lenti 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).

Table 2. Calculation worksheet for scaling *TransIT*[®] Lentivirus System transfections

Starting conditions per milliliter of complete growth medium					
	Per 1 mL		Total culture volume		Reagent quantities
Complex formation solution (e.g. OptiMEM [®])	91.5 µL x			=	_____ mL
Transfer plasmid DNA (1 µg/µL stock)	0.5 µL x		_____ mL	=	_____ µL
Lentivirus Packaging Mix* (0.1 µg/µL stock)	5 µL x			=	_____ µL
<i>TransIT</i> [®] -Lenti Transfection Reagent	3 µL x			=	_____ µL
Total Complex Volume	100 µL				



Transfections with *TransIT*[®]-Lenti Reagent and Lentivirus Packaging Mix were optimized in Freestyle[™] 293-F cells. If using an alternative suspension HEK 293 cell type, determine the best *TransIT*[®]-Lenti Reagent:DNA ratio per cell type by varying the amount of *TransIT*[®]-Lenti Reagent from 2-4 µL per 1 µg total DNA.

*Lentivirus Packaging Mix Powered by MISSION[®] Genomics. MISSION[®] is a registered trademark of Sigma-Aldrich[®] Co. LLC.

Materials Required but Not Supplied

- FreeStyle[™] 293-F cells (Life Technologies[®] Cat. No. R790-07)
- Complete Culture Medium (e.g. FreeStyle[™] F17 + 4mM L-Glutamine + 0.2% Poloxamer 188)
- Nucleic acid (2nd or 3rd generation transfer vector containing GOI)
- Serum-free medium (e.g. Opti-MEM[®] I Reduced-Serum Medium)
NOTE: OptiPRO[™] SFM (Life Technologies, Cat. No. 12309-050) is animal-origin free and can be used for complex formation.
- 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)

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 2–3 x 10⁶ 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 < 95% viable.

2. Incubate cells overnight at appropriate temperature and CO₂ levels (e.g. 37°C, 5–8% CO₂, shaking).

B. Prepare TransIT[®]-Lenti:DNA complexes (Immediately before transfection)

1. Immediately prior to transfection, seed cells at a density of 2 × 10⁶ cells/mL into a transfection culture vessel (e.g. 25 mL per 125 mL Erlenmeyer shake flask).
2. Warm TransIT[®]-Lenti Reagent to room temperature and vortex gently.
3. Place 2.5 mL of serum-free medium (e.g. OptiMEM[®]) in a sterile tube.
4. Add 25 µg total plasmid DNA (i.e. 12.5 µg transfer vector encoding GOI + 12.5 µg Lentivirus Packaging Mix) to the tube containing OptiMEM[®]. Pipet gently to mix completely.
5. Add 75 µL TransIT[®]-Lenti Reagent to the diluted DNA. Pipet gently to mix completely.
6. Incubate at room temperature for 10 minutes to allow transfection complexes to form.



Warm TransIT[®]-Lenti Reagent to room temperature and vortex gently before each use.

C. Distribute the complexes to cells in complete growth medium

1. Add the TransIT[®]-Lenti 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 CO₂ levels (e.g. 37°C, 5–8% CO₂).
3. Incubate cultures for 48 hours prior to virus harvest.



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

D. 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 producing a large batch of the same virus, 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 + 10mM HEPES pH 7.4)
- Lentivirus stock(s) expressing GFP reporter
- *TransducelIT*[™] Reagent (10 mg/mL, Mirus Cat. No. MIR 6620)
- 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 $\geq 40\%$ 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% CO₂ 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 *TransducelIT*[™] 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 *TransducelIT*[™] 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 *TransducelIT*[™] 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 $\leq 5.0 \times 10^7$ TU/mL: Add 1µL, 3µL and 5µL of the lentiviral stock to separate wells.
 - For titers expected to be $\geq 5.0 \times 10^7$ 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% CO₂ 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:

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

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 the transfer vector plasmid DNA.
Suboptimal TransIT®-Lenti:DNA ratio	Determine the best TransIT-Lenti® Reagent:DNA ratio for each cell type. Titrate the volume of TransIT®-Lenti Reagent from 2–4 µL per 1 µg total 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 total 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 TransIT®-Lenti Reagent 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.
Transfection incubation time	Determine the optimal transfection incubation time for each cell type and experiment. Test a range of incubation times (e.g. 24–72 hours). The best incubation time for lentivirus production is 48 hours.
TransIT®-Lenti was not mixed properly	Warm TransIT®-Lenti Transfection Reagent to room temperature and vortex gently before each use.
Precipitate formation during transfection complex formation	During complex formation, scale all reagents according to Tables 1-2 including: serum-free media, TransIT®-Lenti Transfection Reagent, Lentivirus Packaging Mix Powered by MISSION® Genomics and transfer 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*® Nucleic Acid Labeling Kits to label the target plasmid or use Mirus prelabeled *Label IT*® Plasmid Delivery Controls.

To verify efficient transfection, use *TransIT*®-Lenti Reagent to deliver a positive control such as a luciferase, beta-galactosidase or green fluorescent protein (GFP) encoding plasmid.

HIGH CELLULAR TOXICITY

Problem

Solution

Cell density not optimal at time of transfection

Determine optimal cell density for each HEK 293 subtype to maximize transfection efficiency. Use this density to ensure reproducibility. We recommend $\geq 80\%$ confluence at transfection for high lentivirus production in adherent cultures. The recommended cell density for suspension HEK 293-F cells is 2×10^6 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.

Cell morphology has changed

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 HEK 293 cells below passage 30 for optimal recombinant lentivirus production

Transfection complexes and cells not mixed thoroughly after complex addition

Add transfection complexes drop-wise to the cells. Gently rock the culture vessel back-and-forth and from side-to-side to distribute the complexes evenly. Do not swirl or rotate culture vessels containing adherent cells, as this may cause uneven distribution.

Transfection complexes added to cells cultured in serum-free medium

TransIT®-Lenti Transfection Reagent efficiently transfects cells cultured in serum-free medium; however, toxicity may be higher if serum is not present at transfection for cells typically cultured in serum-containing medium. HEK 293 cells adapted to serum-free suspension conditions readily transfect with *TransIT*®-Lenti and no cytotoxicity is observed.

RELATED PRODUCTS

- *TransIT*-VirusGEN® Transfection Reagent
- *TransIT*®-LT1 Transfection Reagent
- *TransIT*®-293 Transfection Reagent
- Ingenio® Electroporation Solution and Kits
- *Label IT*® Plasmid Delivery Controls
- *Label IT*® RNAi Delivery Controls
- *Label IT*® Nucleic Acid Labeling Kits
- MiraCLEAN® Endotoxin Removal Kits



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V1.0 11NOV2025