*Trans*IT®-Lenti Transfection Reagent

Mirus The Transfection Experts

Protocol for MIR 6600, 6603, 6604, 6605, 6606

Quick Reference Protocol, SDS and Certificate of Analysis available at mirusbio.com/6600

INTRODUCTION

Lentivirus is an enveloped, single-stranded RNA virus from the *Retroviridae* family capable of infecting both dividing and non-dividing cells. In conjunction 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.

To produce lentivirus, cells are transfected with packaging plasmids encoding the required *gag*, *pol*, and *rev* structural and regulatory genes as well as a transfer vector encoding the gene of interest (GOI). Replication incompetence is achieved by expressing the minimal virus components on separate plasmids and incorporating the self in-activating (SIN) elements through a major deletion of the 3' long terminal repeat (LTR) within the transfer vector. Essential components are combined with the VSV envelope protein G for a broad virus tropism and increased stability during purification procedures. The lentivirus particles are secreted into the culture medium where they are collected, filtered and frozen into aliquots for subsequent transduction into target cells.

HEK 293T cells are commonly used for lentivirus production due to high transfection efficiencies and protein expression levels; however, the lentivirus platform is often limited by insufficient viral titers requiring concentration before use. To address this limitation, Mirus Bio developed *Trans*IT®-Lenti Transfection Reagent for enhanced delivery of the essential vectors required for higher-titer lentivirus production. With *Trans*IT®-Lenti, greater than 2-fold higher functional titers are achieved over competing transfection reagents.

SPECIFICATIONS

Storage	Store *Trans*IT®-Lenti Transfection Reagent tightly capped at –20°C. ***Before each use***, warm to room temperature and vortex gently.
Product Guarantee	6 months from date of purchase, when properly stored and handled.

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

MATERIALS

Materials Supplied

*Trans*IT®-Lenti Transfection Reagent is supplied in the following formats.

Product No.	Quantity
MIR 6603	1 × 0.3 ml
MIR 6604	1 × 0.75 ml
MIR 6600	1 × 1.5 ml
MIR 6605	5 × 1.5 ml
MIR 6606	10 × 1.5 ml

Materials Required but not Supplied

- 293T cells (e.g. 293T/17 cells, ATCC Cat. No. CRL-11268)
- Appropriate cell culture medium (e.g. DMEM + 10% FBS + 10mM HEPEs pH 7.4)
- Nucleic acid (2nd or 3rd generation packaging plasmids and transfer vector with 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

For Research Use Only.

BEFORE YOU START:

Important Tips for Optimal Lentivirus Production

Mirus recommends using 293T/17 cells (ATCC Cat. No. CRL-11268) for high titer lentivirus production. The suggestions below yield high efficiency plasmid DNA transfection using the *Trans*IT®-Lenti Transfection Reagent.

- **Cell density (% confluence) at transfection.** The recommended cell density for 293T/17 cells is ≥ 80% confluence. Divide cells 18–24 hours before transfection to ensure that the cells are actively dividing and reach the appropriate cell density at the time of transfection.
- **DNA purity.** Use highly purified, sterile, and contaminant-free DNA for transfection. Plasmid DNA preparations that are endotoxin-free and have $A_{260/280}$ 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.
- **Packaging and transfer plasmids.** The *Trans*IT®-Lenti 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.
- **Ratio of *Trans*IT®-Lenti to DNA.** Determine the best *Trans*IT®-Lenti:DNA ratio for each cell type. Start with 3 µl of *Trans*IT®-Lenti per 1 µg of total DNA. Vary the amount of *Trans*IT®-Lenti from 2–4 µl per 1 µg DNA to find the optimal ratio. **Table 1** provides recommended starting conditions based on cell culture vessel size.
- **Complex formation conditions.** Prepare *Trans*IT®-Lenti:DNAcomplexes in serum-free growth medium (e.g. Opti-MEM® I Reduced-Serum Medium).
- **Cell culture conditions.** Culture cells in the appropriate medium, with or without serum. There is no need to perform a medium change to remove the transfection complexes.
- **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 lentivirus titers; therefore, we do not recommend a media change post-transfection.
- **Post-transfection incubation time.** 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 medium during transfection complex formation.

Table 1. Recommended *Trans*IT®-Lenti starting conditions for DNA Transfections

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
Packaging DNA Premix (1 µg/µl stock)	0.13 µl	0.25 µl	0.5 µl	1.0 µl	5 µl	7.5 µl
*Trans*IT®-Lenti Reagent	0.78 µl	1.5 µl	3 µl	6 µl	30 µl	45 µl

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 *Trans*IT®-Lenti need to be pipetted, dilute the reagent in serum-free medium before each use to avoid pipetting errors. ***Do not*** store diluted *Trans*IT®-Lenti.

SECTION I: PLASMID DNA TRANSFECTION

The following procedure describes how to perform plasmid DNA transfections using *Trans*IT®-Lenti Transfection Reagent in 6-well plates. 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®-Lenti, DNA and complete culture medium based on the size of the cell culture vessel (please refer to **Table 1** on Page 2).

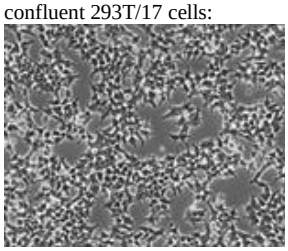
Transient Plasmid Transfection Protocol per Well of a 6-Well Plate

A. Plate cells

1. Approximately 18–24 hours before transfection, plate 293T/17 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% confluent at the time of transfection (see representative image at right).
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 transfection.

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



B. Prepare *Trans*IT®-Lenti:DNA complexes (Immediately before transfection)

1. Warm *Trans*IT®-Lenti 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 the packaging plasmid premix (or individual plasmids) and transfer plasmid encoding the gene of interest (GOI). Mix thoroughly.
4. Transfer 2.0 µg (1 µg packaging plasmid mix + 1 µg transfer plasmid) of the DNA prepared in Step B.3 to the tube containing Opti-MEM® I Reduced-Serum Medium.
5. Pipet gently to mix completely.
6. Add 6.0 µl *Trans*IT®-Lenti to the diluted DNA mixture.
7. Pipet gently to mix completely.
8. Incubate at room temperature for 10 minutes to allow sufficient time for complexes to form.

C. Distribute the complexes to cells in complete growth medium

1. Add the *Trans*IT®-Lenti: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®-Lenti: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 slough off during harvest, spin down 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 in cryogenic tubes and store at -80°C.

SECTION II: LENTIVIRUS TRANSDUCTION AND TITERING PROTOCOL USING GFP REPORTER VIRUS

The following procedure describes how to transduce HEK 293T/17 cells grown in a 24-well tissue culture plate 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

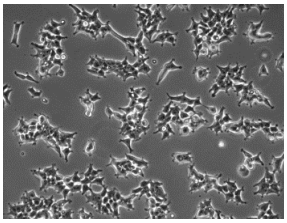
- 293T/17 cells (ATCC Cat. No. CRL-11268)
- Appropriate cell culture medium (e.g. DMEM + 10% FBS + 10mM HEPES pH 7.4)
- Lentivirus stock(s) (GFP reporter virus)
- *Transduce*IT™ Reagent (10 mg/ml, Mirus Cat. No. MIR 6620) or hexadimethrine bromide (Sigma, Cat. No. H9268)
- 24-well tissue culture plate(s)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

A. Plate cells

1. Approximately 18–24 hours before transduction, plate 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.5).
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. Dilute *Transduce*IT™ Reagent or hexadimethrine bromide to a working concentration of 16 µg/ml in pre-warmed complete growth medium (e.g. add 6.25 µl of a 10 mg/ml solution into 10 ml of growth medium).
2. Gently remove half of the medium from each well using a P1000 micropipettor.
3. 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 293T/17, the optimal concentration of *Transduce*IT® or hexadimethrine bromide should be empirically determined.
4. Add dilutions of the lentivirus stock to separate well. 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.
5. Trypsinize and count 2 extra wells of untransduced cells (plated in A.1) to obtain an accurate cell concentration at the time of transduction.
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:

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

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®-Lenti:DNA ratio	Determine the best *Trans*IT®-Lenti:DNA ratio for each cell type. Titrate the *Trans*IT®-Lenti from 2–4 µl 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®-Lenti 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.
*Trans*IT®-Lenti was not mixed properly	Warm *Trans*IT®-Lenti to room temperature and vortex gently before each use.
Precipitate formation during transfection complex formation	During complex formation, scale all reagents according to **Table 1** on Page 2 including: serum-free media, *Trans*IT®-Lenti 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 8). To verify efficient transfection, use *Trans*IT®-Lenti 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	Determine optimal cell density for each HEK 293T subtype to maximize transfection efficiency. Use this density to ensure reproducibility. We recommend ≥ 80% confluence at transfection for high lentivirus titers.
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 293T/17 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 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®-Lenti 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.

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RELATED PRODUCTS

- Ingenio® Electroporation Solution and Kits
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- *Label* IT® Tracker™ Intracellular Nucleic Acid Localization Kits
- MiraCLEAN® Endotoxin Removal Kits
- *Trans*IT-X2® Dynamic Delivery System
- *Trans*IT®-2020 Transfection Reagent
- *Trans*IT®-LT1 Transfection Reagent
- *Trans*IT® Cell Line Specific Transfection Reagents and Kits
- *Trans*IT-siQUEST® Transfection Reagent
- *Trans*IT-TKO® 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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Contact Mirus Bio for additional information.

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