LV-MAX™ Lentiviral Production System
USER GUIDE

For suspension lentiviral production in a chemically defined, serum-free medium

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Revision history: Pub. No. MAN0017000

<table>
<thead>
<tr>
<th>Revision</th>
<th>Date</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.0</td>
<td>14 July 2017</td>
<td>New document</td>
</tr>
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</table>

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## Product information

**Product description** ............................................................. 5

**Contents and storage** ............................................................ 5

**Components of LV-MAX™ Lentiviral Production System** ................. 6
  - Gibco™ Viral Production Cells ............................................. 6
  - LV-MAX™ Production Medium ............................................. 6
  - LV-MAX™ Supplement .......................................................... 6
  - LV-MAX™ Transfection Reagent .......................................... 6
  - LV-MAX™ Enhancer ............................................................. 6

**Required materials not supplied** ............................................. 7

**Related products** ................................................................ 7

## Thaw and establish Gibco™ Viral Production Cells ..................... 8

**Required materials not supplied** ............................................. 8

**Procedural guidelines** .......................................................... 8

**Count suspension cells** .......................................................... 9

**Workflow:** Thaw and establish Gibco™ Viral Production Cells ........ 10

Thaw and establish Gibco™ Viral Production Cells .................................. 11

Cryopreserve Gibco™ Viral Production Cells ..................................... 12

## High throughput lentiviral production in 2 mL 96-deep well block .. 13

**Required materials not supplied** ............................................. 13

**Procedural guidelines** .......................................................... 13

**Optimized transfection conditions** ........................................... 14

**Workflow:** Lentiviral production in 96-deep well block .................. 15

Transfect cells and produce lentiviral vectors ..................................... 16
Product information

IMPORTANT! Before using this product, read and understand the information in the “Safety” appendix in this document.

Product description

The Gibco™ LV-MAX™ Lentiviral Production System is a high-yield transient lentiviral production system based on HEK293F cells adapted to a special chemically defined, serum-free and protein free LV-MAX™ Production Medium in suspension form. The LV-MAX™ Lentiviral Production System provides cells, culture production medium, supplement, transfection reagent and enhancer to produce high titer lentiviral vectors.

Contents and storage

Table 1 LV-MAX™ Lentiviral Production System Starter Kit (Cat No. A35684)

<table>
<thead>
<tr>
<th>Contents</th>
<th>Cat. No.</th>
<th>Amount</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gibco™ Viral Production Cells (1 X 10^7 cells/mL)</td>
<td>A35347</td>
<td>2 × 1 mL</td>
<td>Liquid nitrogen[^1^]</td>
</tr>
<tr>
<td>LV-MAX™ Production Medium</td>
<td>A3583401</td>
<td>1 L</td>
<td>2°C to 8°C; Protect from light</td>
</tr>
<tr>
<td>LV-MAX™ Transfection Kit:</td>
<td>A35346</td>
<td>1 kit</td>
<td>—</td>
</tr>
<tr>
<td>LV-MAX™ Supplement</td>
<td>—</td>
<td>15 mL</td>
<td>2°C to 8°C; Protect from light</td>
</tr>
<tr>
<td>LV-MAX™ Transfection Reagent</td>
<td>—</td>
<td>2 × 0.9 mL</td>
<td>2°C to 8°C</td>
</tr>
<tr>
<td>LV-MAX™ Enhancer</td>
<td>—</td>
<td>12 mL</td>
<td>2°C to 8°C</td>
</tr>
</tbody>
</table>

[^1^] Store the frozen cells in liquid nitrogen until ready to use. Do not store the cells at −80°C.

The LV-MAX™ Lentiviral Production System Starter Kit provides sufficient material for 300 mL lentiviral production volume.

Required culture vessels:

- Three 96-deep well blocks (270 wells filled/1 mL LV production per well. This is includes estimate 10% procedure-related loss)
- Ten to sixty 50 mL conical tubes (5 mL~30 mL LV production)
- Ten 125 mL shaker flasks (30 mL LV production)
Components of LV-MAX™ Lentiviral Production System

The LV-MAX™ Lentiviral Production System is designed to allow high-density of suspension Gibco™ Viral Production Cells to be transient transfected a chemically defined, serum-free medium to produce high titer lentiviral vectors. The system includes Gibco™ Viral Production Cells which are HEK293F cells adapted to serum-free, high-density suspension culture in LV-MAX™ Production Medium; LV-MAX™ Transfection Reagent which efficiently delivers plenti-transfer plasmid and lentiviral packaging mix into high-density Gibco™ Viral Production Cells; LV-MAX™ Supplement which controls cell growth during the lentiviral vectors (LVVs) production; and LV-MAX™ Enhancer which boosts viral producer cells to produce LVVs. Except Viral Production Cells, all of the components of LV-MAX™ Lentiviral Production System are animal origin-free.

**Gibco™ Viral Production Cells**

The Gibco™ Viral Production Cells are a clonal derivative of the original HEK293F cell line and adapted to a special chemically defined, serum-free and protein-free LV-MAX™ Production Medium. Frozen cells are provided and can be thawed directly into LV-MAX™ Production Medium.

Gibco™ Viral Production Cells characteristics:

- Established from primary embryonic human kidney
- Transformed with sheared human adenovirus type 5 DNA
- Expressed the E1A adenovirus gene
- Produce high levels of protein
- Doubling time of ~26 hours
- Reaches maximum cell densities of ~1 x 10⁷ cells/mL in shaker flask culture
- High lentiviral production between cell passage 5-20

**LV-MAX™ Production Medium**

LV-MAX™ Production Medium is a chemically defined, serum-free, protein-free medium, specially developed for growth and transfection of suspension-adapted human embryonic kidney (HEK) 293 cells, requires no additional supplementation, and is ready for use.

**LV-MAX™ Supplement**

LV-MAX™ Supplement is an optimized, chemically defined, serum-free, protein-free, animal origin-free formulation designed to suppress cell growth during transfection and increase lentiviral vector production without compromising the cell viability and interfering cell lentiviral production process.

**LV-MAX™ Transfection Reagent**

LV-MAX™ Transfection Reagent is uniquely designed for co-transfecting multiple plasmids into high density Gibco™ Viral Production Cells cultures with high transfection efficiency and low toxicity.

**LV-MAX™ Enhancer**

LV-MAX™ Enhancer is a proprietary, animal origin-free formulation designed to boost cell LVVs production.
Required materials not supplied

Unless otherwise indicated, all materials are available through thermofisher.com. MLS: Fisher Scientific (fisherscientific.com) or other major laboratory supplier.

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polycarbonate, plain bottom, sterile, vent-up un baffled shaker flasks:</td>
<td></td>
</tr>
<tr>
<td>125 mL</td>
<td>4115-0125</td>
</tr>
<tr>
<td>250 mL</td>
<td>4115-0250</td>
</tr>
<tr>
<td>1 L</td>
<td>4115-1000</td>
</tr>
<tr>
<td>CO₂ resistant orbital shaker</td>
<td>88881101</td>
</tr>
<tr>
<td>Equipment and reagents to determine cell density and viability</td>
<td>MLS</td>
</tr>
<tr>
<td>Opti-MEM™ I Reduced Serum Medium</td>
<td>31985088</td>
</tr>
</tbody>
</table>

Related products

Unless otherwise indicated, all materials are available through thermofisher.com.

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vivid Colors™ pLenti6.3/V5-GW/EmGFP Expression Control Vector</td>
<td>V37006</td>
</tr>
<tr>
<td>ViraPower™ Lentiviral Packaging Mix</td>
<td>K497500</td>
</tr>
<tr>
<td>Countess™ II Automated Cell Counter</td>
<td>AMQAX1000</td>
</tr>
<tr>
<td>Large capacity cell culture incubator controls 37°C, 8% CO₂ and 75–80% humidity</td>
<td>3950 (if applicable for scale-up)</td>
</tr>
</tbody>
</table>
Thaw and establish Gibco™ Viral Production Cells

Required materials not supplied

Unless otherwise indicated, all materials are available through thermofisher.com. MLS: Fisher Scientific (fisherscientific.com) or other major laboratory supplier.

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<td>CO₂ resistant orbital shaker</td>
<td>88881101</td>
</tr>
<tr>
<td>Equipment and reagents to determine cell density and viability</td>
<td>MLS</td>
</tr>
</tbody>
</table>

Procedural guidelines

- All solutions and equipment that come in contact with cells must be sterile
- Gibco™ Viral Production Cells are supplied in a vial containing 1 mL of cells at 1 × 10⁷ cells/mL in 90% LV-MAX™ Production Medium and 10% DMSO.
- Store the frozen cells in liquid nitrogen until ready to use. Do not store the cells at −80°C
- Thaw and transfer cells directly into 125 mL shaker flask with 30 mL pre-warmed 37°C LV-MAX™ Production Medium; no spin and medium change required upon inoculation
- Three days post-thaw, viable cell density should be approximately 1 × 10⁶/mL and cell viability should be around 90%
- By passaging cells 2–3 times, viability should be ≥90%
- Allow freshly thawed cells to recover in culture for 5 passages post-thaw before transfecting
- Inspect cells to ensure round form is maintain for most population and minimal clumping. Stop and contact technical support if you feel there is an issue with your cells at thermofisher.com/support.
- Subculture cells: split cell every 3–4 day culture; 2 times a week (e.g. Monday, Friday)
• For general maintenance of cells, passage Gibco™ Viral Production Cells when they reach a density of \( \sim 3.5\times10^6 \) viable cells/mL, typically every 3–4 days.

• Warm LV-MAX™ Production Medium to room temperature or 37°C before use.

• Discard cells after passage 20.

• Recommended cell density for routine cell culture:

<table>
<thead>
<tr>
<th>Subculture</th>
<th>Seeding density</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 day</td>
<td>( 0.55 \times 10^6 ) viable cells/mL</td>
</tr>
<tr>
<td>4 day</td>
<td>( 0.35 \times 10^6 ) viable cells/mL</td>
</tr>
</tbody>
</table>

• Recommended shake speed and culture volume for cultivation:

<table>
<thead>
<tr>
<th>Production vessel</th>
<th>Shake speed (Orbit diameter)</th>
<th>Vessel size</th>
<th>Cell culture volume range (24%–40% of vessel size)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shaker flask (vented, not baffled)</td>
<td>125 rpm (19 mm)</td>
<td>125 mL</td>
<td>30 mL – 50 mL</td>
</tr>
<tr>
<td></td>
<td>120 rpm (25 mm)</td>
<td>250 mL</td>
<td>60 mL – 100 mL</td>
</tr>
<tr>
<td></td>
<td>95 rpm (50 mm)</td>
<td>1 L</td>
<td>240 mL – 400 mL</td>
</tr>
</tbody>
</table>

**Count suspension cells**

The optimal success of your lentiviral production is based on the cell density. The cell density is dependent on an accurate cell count. Follow these steps to ensure the best cell count.

1. Prepare your pipette aid with 10 mL pipette (keep it in your hand).

2. With your other hand, shake the flask clockwise 3 times.

3. Shake the flask counterclockwise 3 times.

4. Shake the flask back and forth, left and right 3 times for each way.

5. Pipet cells up and down 3 times.

6. Remove 1 mL for cell count.

7. Identify your cell density by counting the cell at least 2 times.
   If your counts vary more than 10% you should continue to count until you have a reliable average cell density.
Workflow: Thaw and establish Gibco™ Viral Production Cells

Thaw cells

1. Monday

Week 1

2. Thursday

Week 2

3. Monday

Week 3

4. Friday

5. Monday

Week 4

6. Friday

7. Monday

Culture cells

Culture cells & prepare cells for production

8. Prepare cells for next day LV production

Follow LV production protocol

Continue subculture
Thaw and establish Gibco™ Viral Production Cells

Week 1 (P0–P1):

1. **Monday** Passage 0: Thaw a vial of Gibco™ Viral Production Cells in 37°C water bath about ~2–3 minutes until only a small ice block remains. Directly transfer all the cells into 125 mL shaker flask with 30 mL LV-MAX™ Production Medium, no spin and medium change required. Incubate the cells in a 37°C incubator with ≥80% relative humidity and 8% CO₂ on an orbital shaker platform (see Table 3 for recommended shaker speed) (P0).

2. **Thursday** Passage 1 (after 3-day culture): Seed cells at a density of 0.35 × 10⁶ cells/mL of total volume 65 mL in a new 250 mL shaker flask (P1).

   **Note:** Three days post-thaw, viable cell density should be approximately 1x10⁶/mL and cell viability should be around 90%.

Week 2 (P2–P3):

3. **Monday** Passage 2 (after 4-day culture): Seed cells at a density of 0.35 × 10⁶ cells/mL of total culture volume 75 mL in a new 250 mL shaker flask (P2).

4. **Friday** Passage 3 (after 4-day culture): Seed cells at a density of 0.55 × 10⁶ cells/mL of total volume 75 mL in a new 250 mL shaker flask (P3).

   **Note:** By Passage 2, viability should be ≥90%.

Week 3 (P4–P5):

5. **Monday** Passage 4 (after 3-day culture): Seed cells at a density of 0.35 × 10⁶ cells/mL of total volume 75 mL in a new 250 mL shaker flask (P4).

6. **Friday** Passage 5 (after 4-day culture): Cells are established and ready for LV production: Seed cells at a density of 0.55 × 10⁶ cells/mL of total volume 300 mL in a 1L shaker flask enabling you to produce 10 × 30 mL of lentivirus next week (P5).

   **Note:** Prepare cells for following week, 300 mL (10 × 30 mL) LV production.

Week 4 (Cells are ready for LV production):

7. **Monday** See Table 2 for recommended seeding density for subculture.

8. Follow production protocol for: 2 mL 96-deep well block, 50 mL conical tube, or shaker flask.
Cryopreserve Gibco™ Viral Production Cells

Gibco™ Viral Production Cells can be frozen directly in 90% fresh LV-MAX™ Production Medium plus 10% DMSO.

1. Bank cells at passage 3 when cell density reaches ~3.5–5.5 × 10⁶ viable cells/mL > 95% viability.

2. Centrifuge the cells at 100 × g (low speed) for 5 minutes to pellet, discard the spent medium.

3. Prepare medium for cryopreservation: e.g. For 10 mL of freezing medium: to 9 mL of LV-MAX™ Production Medium add 1 mL DMSO, mix well and sterilized by 0.2 µm filtration.

4. Gently resuspend the cell pellet in the prepared freezing medium.

5. Dilute the cells to a final density of 1 × 10⁷ viable cells/mL and aliquot 1 mL per cryovial.

6. Freeze the cells at −80°C for one day.

7. Transfer frozen vials to liquid nitrogen for long-term storage.
High throughput lentiviral production in 2 mL 96-deep well block

Required materials not supplied

Unless otherwise indicated, all materials are available through thermofisher.com. MLS: Fisher Scientific (fisherscientific.com) or other major laboratory supplier.

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
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<tbody>
<tr>
<td>Opti-MEM™ I Reduced Serum Medium</td>
<td>31985088</td>
</tr>
<tr>
<td>3 mm orbital shaker</td>
<td>MLS</td>
</tr>
<tr>
<td>2 mL sterile 96-deep well block</td>
<td>MLS</td>
</tr>
<tr>
<td>2 mL sterile 96-deep well block (V bottom)</td>
<td>MLS</td>
</tr>
<tr>
<td>96-well round bottom plate</td>
<td>MLS</td>
</tr>
<tr>
<td>PureLink™ Air Porous Tape</td>
<td>12262010</td>
</tr>
</tbody>
</table>

Procedural guidelines

- Follow the guidelines in “Thaw and establish Gibco™ Viral Production Cells” on page 8 to culture cells
- Warm LV-MAX™ Production Medium to room temperature or 37°C prior to transfection
- LV-MAX™ Supplement, LV-MAX™ Transfection Reagent, and Opti-MEM™ I Reduced Serum Medium can be used at room temperature or cold
- Use 1 mL LV production for each well of 2 mL 96-deep well block
- Set 3 mm orbital shaker to 1250 rpm
- Lentiviral production volume goal is 105 mL for each 96-deep well block (10% excess to cover loss)
- Prepared cell culture is 90% of LV production volume prior to adding DNA/LV-MAX™ Transfection Reagent complex
  - Includes LV-MAX™ Supplement = 5% of 105 mL LV production
- DNA/LV-MAX™ Transfection Reagent complex, Opti-MEM™ I Reduced Serum Medium is 10% of LV production volume
- LV-MAX™ Enhancer is added 5 –14 hours post-transfection
Optimized transfection conditions

- No medium change is required at post-transfection
- One time LVVs harvesting
- Harvested LVVs can be stored in 4°C for 1 day without losing activity and in −80°C freezer for long-term storage
- Each freeze and thaw cycle decreases virus activity by 30−40%

Optimized transfection conditions

- Total Opti-MEM™ I Reduced Serum Medium is 2 × 5% of LV production volume
- Total DNA amount per ml for LV production is 2.5µg/mL (lentiviral packaging mix + lentiviral transfer vector)
- Lentiviral packaging mix : lentiviral transfer vector (w/w) is 3:2
- LV-MAX™ Transfection Reagent is 6 µL/mL of LV production
- LV-MAX™ Enhancer is 4% of LV production volume
Workflow: Lentiviral production in 96-deep well block

1. Prepare cells
2. Plate cells
3. Make complex
4. Transfect cells
5. Add enhancer
6. Harvest LVVs
7. Measure Titer

Day 0

Day 1

Day 3

LV-MAX™ Lentiviral Production System User Guide
Transfect cells and produce lentiviral vectors

Monday morning (Day 0):

1. **Prepare cells:**
   Count and dilute 3-day cultured high density cells:
   - If planning to do LV production the next morning, dilute high density cells to $3.5 \times 10^6$ viable cells/mL.
   - If planning to do LV production the next afternoon, dilute high density cells at $3.0 \times 10^6$ viable cells/mL.
   - Continue culturing cells for another 24 hours or 28 hours.
   
   **Note:** If preparing cells in the afternoon, and plan to do LV production the next morning, dilute cells to $4 \times 10^6$ viable cells/mL. If planning to do LV production the next afternoon, dilute cells to $3.5 \times 10^6$ viable cells/mL.

Tuesday morning (Day 1): Transfect cells and produce LVVs

2. **Plate cells** (one 96-deep well block):
   Count prepared cells from Day 0 and determine cell density.
   In a sterile container:
   a. Add 5.25 mL LV-MAX™ Supplement (5% of 105 mL).
      - One 96-deep well block has 105 mL LV production (96 mL plus 10%)
   b. Calculate and add the required volume of high density cells.
      - Assume optimum cell density for LV production: $4 \times 10^6$ viable cells/mL.
      - Assume the density of Day 0 prepared cells: $6 \times 10^6$ viable cells/mL.
      - Total cells needed for 105 mL LV production $= 4 \times 10^6 / 6 \times 10^6$.
      - Volume of high density cells for one block (105 mL) $= 420 \times 10^6 / 6 \times 10^6$.
      - $= 70$ mL
   
   **Note:** Due to varying cell counting methods, we recommend trying different cell densities, such as $2.5, 3$ and $4 \times 10^6$ viable cells/mL to determine the optimum density for your LV production needs.
   c. Calculate and add volume of fresh LV-MAX™ Production Medium.
      - Volume of fresh medium $= 94.5$ mL (90% of 105 mL) − 5.25 mL (LV-MAX™ Supplement) − volume of high density cells.
      - If high density volume is 70 mL, volume of fresh medium $= 94.5$ mL − 5.25 mL − 70 mL $= 19.25$ mL
   
   d. Mix cells well by pipetting up and down, pour cell culture into a sterile culture reservoir.
   e. Aliquot 900 µL of above prepared cell culture to each well of a 2 mL 96-deep well block.
      - 90% of 1 mL per-well production $= 0.9$ mL above prepared culture cells
   f. Use PureLink™ Air Porous Tapes to seal the deep block and put it on a 3 mm orbital shaker (1250 rpm).
3. **Make complex:**

Make DNA/LV-MAX™ Transfection Reagent complex (10% v/v):
Prepare two 15 mL conical tubes and one 96-well round bottom plate.
Tube-1: Labeled as “DNA”

**Note:** Assume you are screening the coding genes in lentiviral transfer vector

a. Add 5.25 mL of Opti-MEM™ I Reduced Serum Medium (5% x 105 mL).

b. Add 157.5 µg lentiviral packaging mix.
   - 2.5 µg/mL DNA including lentiviral packaging mix + lentiviral transfer vector
   - Lentiviral packaging mix : lentiviral transfer vector (w/w) = 3:2
   - Lentiviral packaging mix 1.5 µg/mL, lentiviral transfer vector 1 µg/mL
   - Lentiviral packaging mix in 105 mL production = 1.5 µg/mL x 105 mL = 157.5 µg

c. Vortex briefly, pour the solution into a reservoir and aliquot 50 µL in each well of 96-well round bottom plate by using a multichannel pipette.

d. Add 1 µg of lentiviral transfer vector to each designed well, triplicated runs for each screening lentiviral transfer vector.

Tube-2: Labeled as “TfxR”

a. Add 5.25 mL of Opti-MEM™ I Reduced Serum Medium (5% x 105 mL).

b. Add 630 µl of LV-MAX™ Transfection Reagent = (6 µl/mL x 105 mL).

c. Vortex briefly, then incubate for 1 minute at room temperature.

d. Immediately pour the TfxR solution into a reservoir.

e. Add 50 µl of diluted LV-MAX™ Transfection Reagent to each well of the 96-well plate containing prepared DNA solution, mix well by pipetting up and down.

f. Incubate the combined solution for 10 minutes at room temperature.

4. **Transfect cells:**

Add 100 µL of the DNA/LV-MAX™ Transfection Reagent complex to each well of prepared cells in the 96-deep well block.

**Note:** DNA/LV-MAX™ Transfection Reagent complex is stable up to 1.5 hours.

5. **Add LV-MAX™ Enhancer:**

Post-transfection ~5–6 hours, add 40 µL of LV-MAX™ Enhancer to each well of 96-deep well block.

**Note:** If you did transfection in the late afternoon, then you will add LV-MAX™ Enhancer early morning the next day.
The optimum time frame for adding LV-MAX™ Enhancer is 5–14 hours post-transfection.
Thursday afternoon (Day 3):

6. **Harvest LVVs:**
   a. Post-transfection (48−55 hours), to harvest LVVs, spin 96-deep well block at 900 × g in a swing bucket centrifuge, for 15 minutes.
   
   b. Aliquot contained LVVs supernatant to several new 96-well round bottom plates.

7. **Measure titer:**
   a. Perform titer measurement on fresh harvested LVVs or store in a −80°C freezer.
   
   b. Measure titer using your own established method or see our recommended protocols under section “Lentiviral titer measurement” on page 33.
Lentiviral production in 50 mL conical tube

Required materials not supplied

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<tbody>
<tr>
<td>Opti-MEM™ I Reduced Serum Medium</td>
<td>31985088</td>
</tr>
<tr>
<td>Sterile 50 mL conical tubes</td>
<td>MLS</td>
</tr>
<tr>
<td>50 mL conical tube holder</td>
<td>MLS</td>
</tr>
<tr>
<td>PureLink™ Air Porous Tape</td>
<td>12262010</td>
</tr>
</tbody>
</table>

Procedural guidelines

- Follow the “Thaw and establish Gibco™ Viral Production Cells“ on page 8 guidelines to culture cells
- This protocol is suitable for producing 5–30 mL of high titer lentiviral vectors
- Warm LV-MAX™ Production Medium to room temperature or 37°C prior to transfection
- LV-MAX™ Supplement, LV-MAX™ Transfection Reagent, and Opti-MEM™ I Reduced Serum Medium can be used at room temperature or cold
- LV production volume 5–30mL
- Fix 50 mL conical tube holder on shaker
- Set orbital shaker (19 mm) at 250 rpm
- Suggested cell density for LV production: $4 \times 10^6$ cells/mL

**Note:** Due to cell counter difference, try 3 cell densities, such as 2.5, 3, $4 \times 10^6$ viable cells/mL to determine the optimum density for your LV production. Due to varying cell counting methods, we recommend trying different cell densities, such as 2.5, 3 and $4 \times 10^6$ viable cells/mL to determine the optimum production density for your LV production needs.

- Prepared cell culture is 90% of LV production volume, which includes LV-MAX™ Supplement (5% of LV production volume).
- DNA/LV-MAX™ Transfection Reagent complex, Opti-MEM™ I Reduced Serum Medium is 10% of LV production volume
- LV-MAX™ Enhancer is added post-transfection 5–14 hours
- No medium change required at post-transfection
- One time LVVs harvesting
- Harvested LVVs can be stored in 4°C for 1 day without losing activity and in, −80°C freezer for long-term storage
- Each freeze and thaw cycle decreases virus activity by 30–40%

**Optimized transfection conditions**

- Total Opti-MEM™ I Reduced Serum Medium is 2 × 5% of LV production volume
- Total DNA amount per ml for LV production is 2.5µg/mL (lentiviral packaging mix + lentiviral transfer vector)
- Lentiviral packaging mix : lentiviral transfer vector (w/w) is 3:2
- LV-MAX™ Transfection Reagent is 6 µL/mL of LV production
- LV-MAX™ Enhancer is 4% of LV production volume
Workflow: Lentiviral production in 50 mL conical tube

1. Prepare cells
2. Dilute cells
3. Make complex
4. Transfect cells
5. Add enhancer
6. Harvest LVVs
7. Measure Titer
Transfect cells and produce lentiviral vectors

Monday morning (Day 0):

1. **Prepare cells:**
   - Count and dilute 3 day cultured high density cells:
     - If planning to do LV production next morning, dilute high density cells to $3.5 \times 10^6$ viable cells/mL
     - If planning to do LV production next afternoon, dilute high density cells at $3.0 \times 10^6$ viable cells/mL
     - Continue culturing cells for another 24 hours or 28 hours
   - **Note:** If preparing cells in the afternoon, and plan to do LV production the next morning, dilute cells to $4 \times 10^6$ viable cells/mL. If planning to do LV production the next afternoon, dilute cells to $3.5 \times 10^6$ viable cells/mL.

Tuesday morning (Day 1): Transfect cells and produce LVVs

2. **Dilute cells** (90% of LV production volume):
   a. Count prepared cells from Day 0 and determine cell density.
   b. Calculate the cell numbers required for desired LV production volume (optimum production cell density $\times$ desired LV production volume = cell numbers required).
   c. Determine the high-density cell volume needed (high density cell volume = cell numbers required/cell density of prepared cells from Day 0).
   - **Note:** Due to varying cell counting methods, we recommend trying different cell densities, such as $2.5, 3$ and $4 \times 10^6$ viable cells/mL to determine the optimum production density for your LV production needs.
   d. In a new 50 mL conical tube:
     - Add 5% (v/v) LV-MAX™ Supplement
     - Add required high density cells
     - Add calculated fresh LV-MAX™ Production Medium
   e. Use PureLink™ Air Porous TapePureLink™ to seal the top of tube, instead of cap.
Example calculation:
Dilute cells for 10 mL LV production
Assumptions:
• Optimum production density is identified at $4 \times 10^6$/mL
• Cell density from Day 0 is $6 \times 10^6$ viable cells/mL

In a 50 mL conical tube:
• Add 0.5 mL of LV-MAX™ Supplement (5% x 10 mL)
• Add 6.67 mL of high density cells = ($4 \times 10^6$/mL x 10 mL)/ $6 \times 10^6$ viable cells/mL
• Add 1.8 mL of fresh LV-MAX™ Production Medium = (90% − 5%) x 10 mL − 6.67 mL

Table 4  Production cell preparation [90% of LV production volume]

<table>
<thead>
<tr>
<th>Production vessel</th>
<th>LV-MAX™ Supplement</th>
<th>Cells with culture medium (85% of PV[1])</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mL conical tube</td>
<td>5% of production volume</td>
<td>Volume of high density cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Optimum production cell density) x PV/counted cell density</td>
</tr>
</tbody>
</table>

[1] LV production volume

3. Make complex:
Prepare DNA/LV-MAX™ Transfection Reagent complex (Total volume 10% v/v):
Prepare 2 tubes.
Tube 1: Labeled as “DNA”
a. Add 5% (v/v) Opti-MEM™ I Reduced Serum Medium
   Note: Opti-MEM™ I Reduced Serum Medium volume is independent of DNA volume; do not subtract DNA volume.
b. Add lentiviral packaging mix and lentiviral transfer vector.
   Note: Total DNA will be 2.5 µg/mL x LV production volume (mL) and the ratio of lentiviral packaging mix: lentiviral transfer vector = 3:2 (w/w). Diluted DNA solution is stable at room temperature for up to 30 minutes.

Tube 2: Labeled as ”TfxR”
a. Add 5% (v/v) Opti-MEM™ I Reduced Serum Medium.
   Note: Opti-MEM™ I Reduced Serum Medium volume is independent of LV-MAX™ Transfection Reagent volume; do not subtract transfection reagent volume.
b. Add LV-MAX™ Transfection Reagent = 6 µl/mL x LV production volume (mL).
c. Vortex briefly and incubate for 1 minute at room temperature.
   Note: The incubation can be 1−2 minutes; do not incubate more than 4 minutes.

LV-MAX™ Lentiviral Production System User Guide 23
d. After 1 minute incubation, combine these two solutions by either way, Tube-1 to Tube-2 or reversely and vortex briefly.

e. Incubate combined solution for 10 minutes at room temperature.

**Note**: DNA/LV-MAX™ Transfection Reagent complex can be stable up to 1.5 hr.

4. Transfect cells:

Directly add DNA/LV-MAX™ Transfection Reagent complex to diluted cells, and pipet up and down to mix.

**Example calculation:**

Make DNA/LV-MAX™ Transfection Reagent complex for 10 mL LV production

Prepare two 1.5 mL microcentrifuge tubes

Tube 1: Label as "DNA"
- Add 0.5 mL of Opti-MEM™ I Reduced Serum Medium (5% x 10 mL)
- Add 15 µg of Lentiviral packaging mix (2.5 µg/mL x 3/5 x 10 mL)
- Add 10 µg of pLenti-transfer plasmid (2.5 µg/mL x 2/5 x 10 mL)
- Briefly vortex to mix

Tube 2: Label as "TfxR"
- Add 0.5 mL of Opti-MEM™ I Reduced Serum Medium (5% x 10 mL)
- Add 60 µl of LV-MAX™ Transfection Reagent (6 µl/mL x 10 mL)
- Briefly vortex to mix
- Incubate at room temperature for 1 minute
- After 1 minute incubation, combine the two solutions by adding Tube-1 diluted DNA to Tube-2 diluted TfxR or reversely
- Incubate at room temperature for 10 minutes

Transfect cells
- After 10 minutes of incubation, add the 1 mL complex to the prepared cells and pipet up and down

| Table 5 | DNA/LV-MAX™ Transfection Reagent complex (10% of LV production volume) |
| Tube-1 (DNA) | Tube-2 (TfxR) | Total complex (Tube-1 + Tube-2) |
| Opti-MEM™ I Reduced Serum Medium | Lentiviral packaging mix: lentiviral transfer vector=3:2 (w/w) 2.5 µg/mL | Opti-MEM™ I Reduced Serum Medium | LV-MAX™ Transfection Reagent |
| 5% of PV | 1.5 µg/mL × PV | 6 µg/mL × PV | 10%[2] of PV |

[1] LV production volume
[2] Final volume has a little more than 10% of production volume, including the amount of LV-MAX™ Transfection Reagent and DNA
5. **Add LV-MAX™ Enhancer:**
   Post-transfection ~5−6 hr, add 4% (v/v) of LV-MAX™ Enhancer to each production 50 mL conical tube.
   
   **Example calculation:** Add 0.4 mL of LV-MAX™ Enhancer to 10 mL LV production.
   
   **Note:** If you did transfection later afternoon, then add LV-MAX™ Enhancer next day, early morning. The optimum time frame for adding LV-MAX™ Enhancer is post-transfection 5−14 hr.

**Thursday afternoon (Day 3): Harvest LVVs and measure titer**

6. **Harvest LVVs:**
   a. Post-transfection 48−55 hr, harvest LVVs by transferring culture medium containing LVVs to proper tubes, spin down cell pellets and collect supernatant.
   
   b. Further remove cell debris by filtration (0.45 µM low protein binding filter). LVVs are now ready for downstream purification and concentration.
   
   c. For immediate downstream utilization, the LVVs can be stored at 4°C for one day. For long-term storage, aliquot and store in −80°C freezer.

- Harvest a small volume of LVVs (e.g. 800 µL of crude LVVs): Transfer 1 mL of LVVs culture to a 1.5 mL microcentrifuge tube, spin the tube in a bench top centrifuge, e.g. Thermo Scientific™ MicroCL 17R, at 13,000 rpm for 5 minutes, pellet down cells and collect 800 µL supernatant.
- Harvest large volume crude LVVs (e.g. 20 mL or more): Transfer cell medium with LVVs to proper tubes, use a centrifuge with swing bucket, spin tubes at 1300 × g for 15 minutes, pellet down cells and collect supernatant, then filter with 0.45 µm low protein binding filter to further remove cell debris.

7. **Measure titer:**
   a. Perform titer measurement on fresh harvested LVs or store in a −80°C freezer and measure titers at a later time.
   
   b. Measure titer using your own established method or see our recommended protocols under section “Lentiviral titer measurement” on page 33.
Lentiviral production in shaker flask

Required materials not supplied

Unless otherwise indicated, all materials are available through thermofisher.com. MLS: Fisher Scientific (fisherscientific.com) or other major laboratory supplier.

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polycarbonate, plain bottom, sterile, vent-up unbaffled shaker flasks:</td>
<td></td>
</tr>
<tr>
<td>125 mL</td>
<td>4115-0125</td>
</tr>
<tr>
<td>250 mL</td>
<td>4115-0250</td>
</tr>
<tr>
<td>1 L</td>
<td>4115-1000</td>
</tr>
<tr>
<td>CO₂ resistant orbital shaker</td>
<td>88881101</td>
</tr>
<tr>
<td>Opti-MEM™ I Reduced Serum Medium</td>
<td>31985088</td>
</tr>
</tbody>
</table>

Procedural guidelines

- Follow the “Thaw and establish Gibco™ Viral Production Cells“ on page 8 guidelines to culture cells
- This protocol is suitable for producing 30 mL – 500 mL high titer lentiviral vectors
- Warm LV-MAX™ Production Medium to room temperature or 37°C prior to transfection
- LV-MAX™ Supplement, LV-MAX™ Transfection Reagent, and Opti-MEM™ I Reduced Serum Medium can be used at room temperature or cold
- LV production volume 24%–50% shaker flask size
- Suggested cell density for LV production: 4 × 10⁶ cells/mL
  
  **Note:** Due to varying cell counting methods, we recommend trying different cell densities, such as 2.5, 3 and 4 × 10⁶ viable cells/mL to determine the optimum production density for your LV production needs.
- Prepared cell culture is 90% of LV production volume, which includes LV-MAX™ Supplement (5% of LV production volume)
- DNA/LV-MAX™ Transfection Reagent complex, Opti-MEM™ I Reduced Serum Medium is 10% of LV production volume
- Add LV-MAX™ Enhancer 5–14 hr post-transfection
- No medium change required at post-transfection
- One time LVVs harvesting
• Harvested LVVs can be stored at 4°C for 1 day without losing activity, and in a −80°C freezer for long-term storage
• Each frozen and thaw cycle decreases 30–40% virus activity

Optimized transfection conditions

• Total Opti-MEM™ I Reduced Serum Medium is $2 \times 5\%$ of LV production volume
• Total DNA amount per ml for LV production is $2.5\mu$g/mL (lentiviral packaging mix + lentiviral transfer vector)
• Lentiviral packaging mix : lentiviral transfer vector (w/w) is 3:2
• LV-MAX™ Transfection Reagent is $6 \mu$L/mL of LV production
• LV-MAX™ Enhancer is 4% of LV production volume

Choose right vessel for LV production

<table>
<thead>
<tr>
<th>Production vessel (vented, no baffle)</th>
<th>Shake speed (orbital diameter)</th>
<th>Vessel size</th>
<th>LV production volume range [24%–50%$^{[1]}$ of vessel size]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shaker flask</td>
<td>125 rpm (19 mm)</td>
<td>125 mL</td>
<td>30–62.5 mL</td>
</tr>
<tr>
<td></td>
<td>120 rpm (25 mm)</td>
<td>250 mL</td>
<td>60–125 mL</td>
</tr>
<tr>
<td></td>
<td>95 rpm (50 mm)</td>
<td>500 mL</td>
<td>120–250 mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000 mL</td>
<td>240–500 mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2000 mL</td>
<td>480–1000 mL</td>
</tr>
</tbody>
</table>

$^{[1]}$ LV production volume can be up to 54% of vessel size, including LV-MAX™ Enhancer. Do not exceed 60%.
Workflow: Lentiviral production in shaker flask

1. Prepare cells
2. Dilute cells
3. Make complex
4. Transfect cells
5. Add enhancer
6. Harvest LVVs
7. Measure Titer
Transfect cells and produce lentiviral vectors

Monday morning (Day 0):

1. **Prepare cells:**
   - Count and dilute 3-day cultured high density cells:
     - If planning to do LV production next morning, dilute the high density cells to $3.5 \times 10^6$ viable cells/mL.
     - If planning to do LV production next afternoon, dilute the high density cells to $3.0 \times 10^6$ viable cells/mL.
     - Continue culturing cells for another 24–28 hours.
   - **Note:** If preparing cells in afternoon, and plan to do LV production the next morning, dilute cells to $4 \times 10^6$ viable cells/mL. If planning to do LV production next afternoon, dilute cells to $3.5 \times 10^6$ viable cells/mL.

Tuesday morning (Day 1): Transfect cells and produce LVVs:

2. **Dilute cells (90% of LV production volume):**
   a. Count prepared cells from Day 0 and determine cell density.
   b. Calculate the cell numbers required for desired LV production volume (optimum production cell density $\times$ desired LV production volume = cell numbers required).
   c. Determine the high density cell volume needed (high density cell volume = cell numbers required/cell density of prepared cells from Day 0).
      - Due to varying cell counting methods, we recommend trying different cell densities, such as $2.5, 3$ and $4 \times 10^6$ viable cells/mL to determine the optimum production density for your LV production needs.

   In a new production vessel (Shaker Flask):
   - Add 5% (v/v) LV-MAX™ Supplement.
   - Add required high density cells.
   - Add calculated fresh LV-MAX™ Production Medium.
Example calculation:
Dilute cells for 30 mL LV production
Assumptions:
• Optimum production density is identified at $4 \times 10^6$/mL
• Cell density from Day 0 is $6 \times 10^6$ viable cells/mL
In a 125 mL flask:
• Add 1.5 mL of LV-MAX™ Supplement (5% x 30 mL)
• Add 20 mL of high density cells = $(4 \times 10^6$/mL x 30 mL)/ $6 \times 10^6$ viable cells/mL
• Add 5.5 mL of fresh LV-MAX™ Production Medium = (90% − 5%) x 30 mL − 20 mL

Table 6  Production cell preparation (90% of LV production volume)

<table>
<thead>
<tr>
<th>Production vessel</th>
<th>LV-MAX™ Supplement</th>
<th>Cells with culture medium (85% of PV[1])</th>
</tr>
</thead>
</table>
| Shaker flask      | 5% of production volume | Volume of high density cells
[Optimum production cell density] x PV/ counted cell density | Volume of fresh LV-MAX™ Production Medium
85% PV − volume of high density cells |

[1] LV production volume

3. Make complex:
Prepare DNA/LV-MAX™ Transfection Reagent complex (Total volume 10% v/v):
Prepare two tubes.
Tube 1: Labeled as "DNA"
   a. Add 5% (v/v) Opti-MEM™ I Reduced Serum Medium.
      Note: Opti-MEM™ I Reduced Serum Medium volume is independent of DNA volume (do not subtract DNA volume).
   b. Add lentiviral packaging mix and lentiviral transfer vector.
      Note: Total DNA will be 2.5 µg x LV production volume (mL) and lentiviral packaging mix and lentiviral transfer vector = 3:2 (w/w).
      Diluted DNA solution can be stable at room temperature up to 30 minutes.
Tube 2: Labeled as "TfxR"
   a. Add 5% (v/v) Opti-MEM™ I Reduced Serum Medium.
      Note: Opti-MEM™ I Reduced Serum Medium volume is independent of transfection reagent volume (do not subtract transfection reagent volume).
   b. Add LV-MAX™ Transfection Reagent = 6 µL/mL x LV production volume.
   c. Vortex briefly and incubate for 1 minute at room temperature.
   d. After 1 minute combine these two solutions by either way, Tube-1 to Tube-2 or reversely, and vortex briefly.
e. Incubate combined solution for 10 minutes at room temperature.

**Note:** DNA/LV-MAX™ Transfection Reagent complex can be stable up to 1.5 hours.

4. **Transfect cells:**

After 10 minutes of incubation, directly add DNA/LV-MAX™ Transfection Reagent complex to diluted cells, and shake the flask to mix.

**Example calculation:**

Make DNA/LV-MAX™ Transfection Reagent complex for 30 mL LV production

Prepare two 15 mL conical tubes:

**Tube 1:** Label as “DNA”
- Add 1.5 mL of Opti-MEM™ I Reduced Serum Medium (5% × 30 mL)
- Add 45 μg of lentiviral packaging mix (2.5 μg/mL × 3/5 × 30 mL)
- Add 30 μg of lentiviral transfer vector (2.5 μg/mL × 2/5 × 30 mL)
- Briefly vortex to mix

**Tube 2:** Label as “TfxR”
- Add 1.5 mL of Opti-MEM™ I Reduced Serum Medium (5% x 30 mL)
- Add 180 μl of LV-MAX™ Transfection Reagent (6 μl/mL x 30 mL)
- Briefly vortex to mix
- Incubate at room temperature for 1 minute
- After 1 minute incubation, combine the two solutions by adding Tube-1 diluted DNA to Tube-2 diluted TfxR or reversely.
- Incubate at room temperature for 10 minutes.
- After the 10 minute incubation, add the 3 mL complex to the prepared cells and shake to mix.

**Table 7** DNA/LV-MAX™ Transfection Reagent complex (10% of LV production volume)

<table>
<thead>
<tr>
<th></th>
<th>Tube-1 (DNA)</th>
<th>Tube-2 (TfxR)</th>
<th>Total complex (Tube-1 + Tube-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opti-MEM™ I Reduced Serum Medium</td>
<td>lentiviral packaging mix: lentiviral transfer vector=3:2 (w/w) 2.5 μg/mL</td>
<td>Opti-MEM™ I Reduced Serum Medium</td>
<td>LV-MAX™ Transfection Reagent</td>
</tr>
<tr>
<td></td>
<td>lentiviral packaging mix (1.5 μg/mL)</td>
<td>lentiviral transfer vector (1 μg/mL)</td>
<td></td>
</tr>
<tr>
<td>5% of PV[1]</td>
<td>1.5 μg/mL × PV</td>
<td>1 μg/mL × PV</td>
<td>5% of PV</td>
</tr>
</tbody>
</table>

[1] LV production volume
[2] Final volume has a little more than 10% of production volume, including the amount of LV-MAX™ Transfection Reagent and DNA
5. **Add LV-MAX™ Enhancer.**
Post-transfection ~5−6 hr, add 4% (v/v) of LV-MAX™ Enhancer to each production flask.

**Example calculation:** Add 1.2 mL of LV-MAX™ Enhancer to 30 mL LV production.

**Note:** If you did transfection in the late afternoon, then add LV-MAX™ Enhancer early morning the next day. The optimal time frame for adding LV-MAX™ Enhancer is 5–14 hr post-transfection.

---

**Thursday afternoon (Day 3): Harvest LVVs and measure titer**

6. **Harvest LVVs:**
   a. Post-transfection 48−55 hr, harvest LVVs by transferring culture medium containing LVVs to proper tubes, spin down cell pellets and collect supernatant.

   b. Further remove cell debris by filtration (0.45 µm low protein binding filter). LVVs are ready for downstream purification and concentration.

   c. For immediate downstream utilization, the LVVs can be stored at 4°C for one day. For long-term storage, aliquot and store in a −80°C freezer.
      - Harvest a small volume of LVVs (e.g. 800 µl of crude LVVs): Transfer 1 mL of LVVs culture to a 1.5mL microcentrifuge tube, spin the tube in a bench top centrifuge, such as Thermo Scientific™ Micro 17R, or other similar centrifuges, at 13,000 rpm for 5 minutes, pellet cells and collect 800 µL supernatant. Aliquot and store at −80°C.
      - Harvest large volume crude LVVs (e.g. 20 mL or more): Transfer cell medium with LVVs to proper tubes, use a centrifuge with swing bucket, spin tubes at 1300 × g for 15 minutes, pellet down cells and collect supernatant, then go through 0.45 µm low protein binding filter for further removing cell debris.

7. **Measure titer:**
   a. Perform titer measurement on fresh harvested LVVs or store in −80°C freezer and measure titers at a later time.

   b. Measure titer using your own established method or see our recommended protocols under section “Lentiviral titer measurement” on page 33.
Guidelines for lentiviral titer measurement

- Thaw virus on ice prior to use, do not use any acceleration method
- Virus decreases 30% potency with each freeze/thaw cycle
- Mix virus by tapping or flipping tube; do not vortex and avoid vigorously mix
- IMPORTANT! Handling of lentivirus must be performed as per institutional guidelines. All materials should be treated with a 10% bleach solution prior to disposal

Titer by GFP+

Unless otherwise indicated, all materials are available through thermofisher.com. MLS: Fisher Scientific (fisherscientific.com) or other major laboratory supplier.

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT1080 cell line</td>
<td>ATCC, CCL-121</td>
</tr>
<tr>
<td>DMEM, high glucose, GlutaMAX™ Supplement, pyruvate[1]</td>
<td>10569010</td>
</tr>
<tr>
<td>96-well culture plates</td>
<td>MLS</td>
</tr>
<tr>
<td>96-well round bottom plates</td>
<td>MLS</td>
</tr>
<tr>
<td>Large centrifuge for spinning culture plate</td>
<td>MLS</td>
</tr>
<tr>
<td>TrypLE™ Express Enzyme (1X), no phenol red</td>
<td>12604013</td>
</tr>
<tr>
<td>DPBS, no calcium, no magnesium</td>
<td>14190250</td>
</tr>
</tbody>
</table>

[1] Add 10% FBS to make culture medium for HT1080
[2] Stock solution: 10 mg/mL in sterile H₂O
Lentiviral titer measurement

Titer by GFP+  

GFP titer

This workflow is tailored for a 96-well plate format for high throughput flow analysis.

Day 1. (Morning)

1. 4 hours before infecting HT1080 cells, seed a 96-well culture plate with HT1080 cells at a density of 7000 cells/well in 100uL of culture media (~30% confluent at time of infection).

2. Remove LVVs from −80°C, thaw on ice 2 hours prior to performing titering assay. Don’t accelerate the LVVs thawing process.

3. Freshly prepare virus dilutions as follows:
   a. Prepare dilution medium: 15 mL of fresh culture medium + 12 µL of 10 mg/mL Polybrene (Final concentration of 8 µg/mL), vortex to combine.
   b. In a 96-well round bottom plate: add 135 µL of above prepared dilution medium to each well.
   c. Add 15 µL of Sample 1 (S1) of lentiviral supernatant (or concentrated viral aliquot) to each well in Row-1, total volume 150 µL in each well, quadruple measurement for each sample.
   d. Gently mix well by pipetting.
   e. Continue a serial dilution while changing the pipette tips for each row (using a multichannel pipette if available).
      Transfer 15 µL from Row-1 to Row-2, mix well (100-fold dilution).
      Transfer 15 µL of Row-2 to Row-3, mix well (1000-fold dilution).
      Transfer 15 µL of Row-3 to Row-4, mix well (10,000-fold dilution).
      If your virus was concentrated, you may need more dilutions.

4. To infect cells, remove culture media from HT1080 cells, and infect by transferring 100 µL of the prepared diluted virus to each corresponding well (using a multichannel pipette if available).

5. Spin the 96-well infected cell plate at 900 × g at room temperature for 30 minutes.

6. Incubate the infected cell plate overnight.

Day 2. (Morning)

7. Remove medium from infected cell plate, and replace with fresh HT1080 culture medium (without Polybrene).

8. Incubate cells for an additional 3 days.
Day 5.

9. Remove medium from each well, add 150 µL trypsinization medium (75% TrypLE™ + 25% DPBS mixture) to each well.

10. Incubate cell plate for ~15 minutes in 37°C incubator.

11. Check cells under microscope to ensure cells are rounded up. Pipet up and down by multichannel pipette to detach cells from culture plate bottom.

12. Run flow cytometry.

Calculate lentiviral titer

1. To calculate the titer in units TU/mL, determine appropriate dilution factor to use based on %GFP positivity.
   The desired infection range is 1−20% of GFP, over 20% of GFP in calculation will be underestimated LVV titer.

2. Use the following formula:
   \[ \text{Titer} = \left( F \times \frac{C}{V} \right) \times D \]

   \( F \) = frequency of GFP+ cells (%GFP+ cells/100)
   \( C \) = cell number per well at time of transduction (7000 cells)
   \( V \) = volume of inoculum in mL (0.1mL)
   \( D \) = lentivirus dilution factor

   **Example: [Based on 96-well protocol]**
   - Lentivirus dilution: \(10^2\)  Percent EmGFP positive cells: 96%
   - Lentivirus dilution: \(10^3\)  Percent EmGFP positive cells: 65%
   - Lentivirus dilution: \(10^4\)  Percent EmGFP positive cells: 18%

   **Note:** \(10^4\) is chosen for the calculation because the % GFP positive cell volume falls within the desired 1−20% range.

   \( F = 18/100 \)
   \( C = 7000 \) (cell # at time of infection)
   \( V = 0.1 \) (100 µL medium)
   \( D = 10^4 \)

   Calculation is as follows:
   \[ \text{Titer} = \left( 0.18 \times \frac{7000}{0.1} \right) \times 10^4 = 1.26 \times 10^8 \text{ TU/mL} \]
Titer by antibiotic selection

Required materials not supplied

Unless otherwise indicated, all materials are available through thermofisher.com. MLS: Fisher Scientific (fisherscientific.com) or other major laboratory supplier.

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
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<tbody>
<tr>
<td>HT1080 cell line</td>
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</tr>
<tr>
<td>DMEM, high glucose, GlutaMAX™ Supplement, pyruvate[1]</td>
<td>10569010</td>
</tr>
<tr>
<td>Crystal violet</td>
<td>Fisher Scientific™, ICN15251150</td>
</tr>
<tr>
<td>6-well culture plates</td>
<td>140675</td>
</tr>
<tr>
<td>TrypLE™ Express Enzyme (1X), no phenol red</td>
<td>12604013</td>
</tr>
</tbody>
</table>

[1] Add 10% FBS to make culture medium for HT1080
[2] Stock solution: 10 mg/mL in sterile H₂O and filter through 0.2 µm filter
[3] Selection medium: culture medium with Blasticidin S HCl, 10 μg/mL final concentration (the selection medium based on plenti transfer plasmid back bone selection marker)
[4] Select culture medium final concentration 10 μg/mL

Antibiotic titer

Day 1 (Morning) – Infect HT1080 cells

1. 4 hours before infecting cells, seed a 6-well plate with HT1080 cells at a density of 210,000 cells/well in 2 mL of culture media (~30% confluent at time of infection).

2. Remove LVVs from −80°C, thaw on ice two hours prior to performing titering assay.

   No not accelerate the thawing process.

3. Freshly prepare virus dilutions as follows:
   a. Prepare dilution medium: in a 50 mL conical tube, add 20 mL of fresh HT1080 culture medium and 16 μL of 10 mg/mL Polybrene (final concentration of 8 μg/mL), vortex to mix.
   b. For a viral sample, prepare seven microcentrifuge tubes and label them as T1, T2 to T7.
c. Add 180 µL of prepared dilution medium to T1 and 900 µL to T2 through T7.

![Diagram showing dilution process]

<table>
<thead>
<tr>
<th>Medium</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>180 µL</td>
<td>10</td>
</tr>
<tr>
<td>900 µL</td>
<td>10^2</td>
</tr>
<tr>
<td>900 µL</td>
<td>10^3</td>
</tr>
<tr>
<td>900 µL</td>
<td>10^4</td>
</tr>
<tr>
<td>900 µL</td>
<td>10^5</td>
</tr>
<tr>
<td>900 µL</td>
<td>10^6</td>
</tr>
<tr>
<td>900 µL</td>
<td>10^7</td>
</tr>
</tbody>
</table>

d. Add 20 µL of a viral sample, crude lentivirus or concentrated viral aliquot to T1 (10 fold dilution), gently mix by tapping or flipping the tube.

e. Use a new tip to transfer 100 µL of T1 dilution to T2, inverting to mix (1 × 10^2 fold dilution).

f. Use a new tip to transfer 100 µL of T2 dilution to T3, inverting to mix (1 × 10^3 fold dilution).

g. Repeat above step to finish the rest dilutions, T4 to T7.

**Note:** If your virus was concentrated, you may need more dilutions.

4. Infect HT1080 cells.

a. Remove culture medium from prepared HT1080 cell in the 6-well plate, add 1500 µL of prepared dilution medium to A1, A2, A3, B1, B2 and 2000 µL to B3 as negative control (‘Neg Ctrl’).

b. To infect HT1080 cells in the 6-well plate, follow the LVV infection grid in figure, transferring 500 µL of the prepared diluted virus starting with T3, into each respective well.

![LVVs infection grid]

![Final viral dilution]

5. Mix the diluted virus by moving infected 6-well plate left, right, backward and forward several times.

6. Spin the infected cell plate at 900 × g force (using a swinging bucket centrifuge) at room temperature for 30 minutes.

7. Incubate the infected cell plate overnight.
Day 2 – Change medium and incubate cells

8. Remove medium, and replace with fresh HT1080 culture medium 2mL/well (without Polybrene).

Note: When removing medium and adding fresh medium always start from NegCtrl and work backwards to minimize the amount of virus carried over.

9. Incubate cells for one additional day.

Day 4 – Start antibiotic selection

10. Prepare HT1080 culture medium with antibiotic, such as Blasticidin at final 10 µg/mL or Puromycin at final 1 µg/mL.

Note: You might use other antibiotics based on your pLenti-transfer back bone selection marker.

11. Remove each well of 2 mL culture medium and replace 2 mL above prepared selection medium and incubate.

12. Change and replace 2 mL of fresh selection medium every two days.

13. Repeat above two steps until ‘NegCtrl’ well has no cells left.

Estimate 10 day process.

Day 13 – Crystal Violet stain

14. Prepare the Crystal Violet stain working solution: 1% Crystal Violet in a 10% ethanol/H2O solution.

15. Remove antibiotic selection medium from the 6-well plate and wash each well with 2 mL of PBS.

16. Add 1 mL of prepared Crystal Violet staining solution to stain wells.

17. Incubate plate for 20 minutes at room temperature.

18. After 20 minutes incubation, remove the staining solution from 6-well plate (store solution for future use).

19. Wash stained 6-well plate with water several times until clear background appears.

Calculate viral titer

1. Visually count the number of colonies stained colonies per well.

2. Use the following formula to calculate the titer (Transforming Units/mL) of the viral stock:

\[ \text{TU/mL} = \frac{\# \text{ of discrete colonies} \times \text{dilution factor}}{\text{Volume of inoculum}} \]

Example:
Count of 30 plaques stained in the 4 × 10^6 dilution well (B-1) and 5 plaques stained in 4 × 10^7 dilution well (B-2)
Volume of diluted virus: 2 mL
Titer = \( \frac{30 \times 4 \times 10^6 + 5 \times 4 \times 10^7}{2} \) = 8 × 10^7 TU/mL
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- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
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**WARNING! GENERAL CHEMICAL HANDLING.** To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

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- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer’s cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.
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