Lentiviral Production and Infection Protocol

Complete workflow from transfection to viral transduction

Overview

This protocol describes lentiviral production using HEK293T packaging cells and subsequent infection of target cells. Lentiviral vectors can transduce both dividing and non-dividing cells, making them ideal for stable gene transfer in a wide range of cell types.

Workflow Overview:

- **Day 0:** Plate 293T cells
- **Day 1:** Transfection
- **Day 2:** Media change
- **Day 3-4:** Virus collection
- **Day 4-5:** Virus concentration (optional)
- **Day 5+:** Target cell infection
- **Day 7-8:** Selection or sorting

Applications

- Stable gene overexpression
- shRNA/CRISPR knockdown
- Reporter gene expression
- Gene editing delivery
- Cell line engineering
- Primary cell transduction

Key Components

- **Transfer plasmid:** Contains your gene of interest
- **Packaging plasmid:** pSPAX2 or cDNL (gag-pol)
- **Envelope plasmid:** pMD2.G or VSVG

- **293T cells:** Virus-producing cells
- **Transfection reagent:** Delivers DNA to cells

Biosafety Considerations

- Lentiviral work typically requires BSL-2 containment
- Check institutional biosafety guidelines and obtain IBC approval
- VSV-G pseudotyped lentivirus has broad tropism - handle carefully
- Work in biosafety cabinet for all virus handling steps
- Decontaminate all materials with 10% bleach before disposal
- Never pipette by mouth - use mechanical pipettors
- Wear appropriate PPE: lab coat, gloves, eye protection

Required Materials

Cells & Plasmids

- HEK293T cells (ATCC CRL-3216)
- Target cells for infection
- **Plasmids (per 10cm plate):**
  - • Transfer plasmid: 10-20 µg
  - • pSPAX2 (Addgene #12260): 7.5 µg
  - • pMD2.G (Addgene #12259): 2.5 µg
  - OR cDNL: 4.5-15 µg + VSVG: 0.5-2 µg

Reagents

- DMEM high glucose
- Fetal bovine serum (FBS)
- Penicillin/Streptomycin (optional for transfection)
- Poly-L-lysine solution (0.01%)
- Opti-MEM or serum-free medium
- PBS (phosphate buffered saline)
- 0.45 µm syringe filters (PES or PVDF)
- Polybrene (hexadimethrine bromide), 8 mg/mL stock

Transfection Reagent Options

- **JetPRIME** (Polyplus) - Recommended
- **FuGENE HD** or **X-tremeGENE** (Promega)
- **Lipofectamine 2000/3000** (Thermo Fisher)
- **PEI** (polyethylenimine) - Low cost option
- **Calcium phosphate** - Traditional method

Concentration Reagents (Optional)

- **PEG Precipitation:**
  - • PEG-8000 solution
- **Ultracentrifugation:**
  - • Beckman ultracentrifuge tubes
  - • Ultracentrifuge access
- **Commercial kits:**
  - • Lenti-X Concentrator (Takara)

Equipment

- Biological safety cabinet (BSC)
- CO_2 incubator (37°C, 5% CO_2)
- Centrifuge (benchtop and ultracentrifuge)
- Hemocytometer or cell counter
- 10 cm tissue culture dishes
- 6-well or 12-well plates (for small scale)
- 15 mL and 50 mL conical tubes
- Cryovials for virus storage
- -80°C freezer

Media Recipes

293T Growth Medium:

- DMEM high glucose
- 10% FBS
- 1% Pen/Strep (optional)

Transfection Medium:

- DMEM high glucose
- 10% FBS
- No antibiotics

Select Transfection Method:

JetPRIME (Recommended) | FuGENE/X-tremeGENE | Generic Protocol

Virus Production - JetPRIME Method

> **Scale options: 10cm plate (standard) or 6-well/12-well (small scale)**

Day 0: Plate 293T Cells

Mark Done

- **Poly-L-lysine coating:** Add 5 mL poly-L-lysine to 10cm dish, incubate 30 min at 37°C
- Aspirate poly-L-lysine (can be reused 2-3 times)
- **10cm plate:** Plate 3-8 × 10^6 293T cells in 10 mL DMEM + 10% FBS
- **6-well plate:** Plate 150,000 cells/well in 1 mL medium
- **12-well plate:** Plate 75,000 cells/well in 500 µL medium
- Target: 60-70% confluent on Day 1 (transfection day)
- Incubate overnight at 37°C, 5% CO_2

Day 1: Transfection

Mark Done

- Change medium to fresh DMEM + 10% FBS (no antibiotics)
- **Prepare transfection mix per plate:**

10cm Plate Recipe:

• 500 µL JetPRIME buffer
• 20 µg transfer plasmid (your gene of interest)
• 15 µg cDNL (packaging plasmid)
• 2 µg VSVG (envelope plasmid)
• Vortex, spin down briefly
• Add 10 µL JetPRIME reagent per plate
• Vortex, spin down, incubate 10 min at RT

6-well Plate Recipe:

- 200 µL JetPRIME buffer
- 2 µg total DNA
- 4 µL JetPRIME reagent
- Incubate 10 min at RT

- Add transfection mix dropwise to cells, distribute evenly
- Incubate 4-12 hours (no longer than 12h to avoid toxicity)

Day 2: Media Change

Mark Done

- Aspirate transfection medium
- Wash once with PBS (optional)
- Add fresh DMEM + 10% FBS: 15 mL for 10cm plate, 1-2 mL for 6-well
- Return to incubator

Day 3: First Collection

Mark Done

- Collect medium into 50 mL conical tube
- Store at 4°C if collecting multiple batches
- Add 10-15 mL fresh medium back to plates
- Return to incubator for ~36 hours

Day 4: Second Collection (Optional)

Mark Done

- Collect medium and pool with Day 3 collection
- Total volume: typically 20-30 mL per 10cm plate
- Discard 293T cells (decontaminate with bleach)

Day 4-5: Clarification

Mark Done

- Centrifuge pooled virus at 300-500 × g for 5 min to pellet cell debris
- Transfer supernatant to new tube
- Filter through 0.45 µm syringe filter (work in BSC)
- Virus is now ready for concentration (optional) or direct use
- Aliquot and store at -80°C (stable for months)

Virus Production - FuGENE/X-tremeGENE Method

Day 0: Plate 293T Cells

Mark Done

- Plate 1.5 × 10^6 293T cells per 10cm dish in DMEM + 10% FBS
- Target: 60-70% confluent on Day 1

Day 1 AM: Media Change

Mark Done

- Aspirate growth medium
- Add 15 mL transfection medium (DMEM + 10% FBS, no antibiotics)
- Return to incubator

Day 1 PM: Transfection

Mark Done

- **Prepare master mix per 10cm plate:**

  • 1 mL Opti-MEM
  • 10 µg transfer plasmid
  • 7.5 µg pSPAX2 (packaging plasmid)
  • 2.5 µg pMD2.G (envelope plasmid)
  • Mix gently, add 60 µL FuGENE X-tremeGENE
  • Incubate 20-30 min at room temperature

- Add transfection mix dropwise to cells
- Swirl plate gently to distribute

Day 2: Media Change

Mark Done

- Aspirate supernatant
- Add 10-12 mL collection medium (DMEM + 10% FBS)

Day 3 & 4: Virus Collection

Mark Done

- Collect supernatant into 50 mL tube, store at 4°C
- Add fresh 10-12 mL medium back to plate
- Repeat collection on Day 4, pool with Day 3 collection
- Centrifuge 300 × g for 5 min, filter through 0.45 µm
- Proceed to concentration or store at -80°C

Virus Production - Generic Protocol

> This protocol can be adapted to various transfection reagents. Follow manufacturer's instructions for DNA:reagent ratios.

General Workflow

1. **Day 0:** Plate 293T cells at 60-70% confluency for next day
2. **Day 1:** Transfect with transfer + packaging + envelope plasmids
3. **Day 2:** Change to fresh collection medium
4. **Day 3:** First virus collection, replace with fresh medium
5. **Day 4:** Second virus collection (optional), pool collections
6. **Day 5:** Clarify by centrifugation and filtration

Key Parameters

- **Cell density:** 60-70% confluent at transfection
- **Total DNA:** 10-20 µg per 10cm plate
- **Plasmid ratio:** Transfer : Packaging : Envelope = 4:3:1 to 8:7.5:2.5
- **Collection time:** 48-72 hours post-transfection
- **Peak titer:** Usually Day 3 collection

Select Concentration Method:

Unconcentrated (Easiest) | PEG Precipitation | Ultracentrifugation

Unconcentrated Virus (Direct Use)

> **Simplest method - use filtered virus directly**
>
> Best for: High-expressing constructs, easily transduced cells, quick experiments

- After clarification (300 × g spin) and 0.45 µm filtration, virus is ready to use
- Typical volume: 3 mL virus supernatant per 1-2 × 10^6 target cells
- Add polybrene (8 µg/mL final) to enhance infection
- Store aliquots at -80°C (avoid freeze-thaw cycles)
- Titer: Usually 10^5-10^6 TU/mL

PEG Precipitation Concentration

Concentrates virus 10-50× without special equipment

Best for: Labs without ultracentrifuge, moderate titer needs

Protocol:

1. Clarify virus: spin 300 × g for 5 min, transfer supernatant to new tube
2. Add PEG-8000 solution at 1:3 ratio (virus:PEG)
3. Example: 16 mL virus + 6-8 mL PEG solution
4. Mix by inverting (do not vortex)
5. Incubate at 4°C for 2 hours (or overnight) with rocking
6. Centrifuge at 1,500-1,600 × g for 1 hour at 4°C
7. Carefully remove supernatant (virus pellet at bottom)
8. Resuspend pellet in 1 mL Opti-MEM by pipetting gently
9. Aliquot 200 µL into cryovials, store at -80°C

Expected concentration: ~20-50× original titer

Ultracentrifugation Concentration

Highest concentration (100-1000×) and purity

Best for: High-titer needs, difficult-to-transduce cells, in vivo experiments

Protocol:

1. Pre-clarify virus: centrifuge 300-500 × g for 10 min at 4°C
2. Transfer supernatant to Beckman ultracentrifuge tube
3. Balance tubes precisely (within 0.1 g)
4. Ultracentrifuge: **25,000 RPM for 1.5 hours at 4°C**
5. Carefully aspirate supernatant (virus pellet may not be visible)
6. Resuspend pellet in 200-500 µL Opti-MEM or PBS
7. Incubate on ice 1-2 hours to fully resuspend
8. Aliquot and store at -80°C

Ultracentrifuge Settings:

- Speed: 25,000 RPM (or 50,000-100,000 × g)
- Duration: 1.5 hours
- Temperature: 4°C
- Brake: Slow

Expected concentration: 100-1000× original titer (10^7-10^9 TU/mL)

Target Cell Infection (Transduction)

> **Spinoculation method enhances infection efficiency**
>
> Centrifugation brings virus particles into contact with target cells

Day 0: Prepare Target Cells

Mark Done

- Ensure target cells are in log-phase growth
- Viability should be >95%
- Count cells and prepare appropriate number for infection

Day 1: Infection with Spinoculation

Mark Done

- Count 1-2 × 10^6 target cells into 15 mL tube
- Centrifuge and aspirate supernatant
- **For unconcentrated virus:** Add 3 mL filtered virus
- **For concentrated virus:** Add 20-50 µL virus in 2-3 mL medium
- Add polybrene to **final concentration 8 µg/mL**
- Mix gently
- **Spinoculate:** Centrifuge at 3,000 RPM (900 × g) for 1.5 hours at 32°C
- After spin, resuspend cells and transfer to 6-well plate
- Incubate at 37°C

4 Hours Post-Infection

Mark Done

- Add 2-3 mL fresh medium (appropriate for your cells - RPMI, DMEM, etc.)
- Do not remove virus-containing medium
- Dilutes polybrene and provides fresh nutrients

Day 2: Media Change

Mark Done

- Centrifuge cells and aspirate virus-containing medium
- Resuspend in fresh complete medium
- Return to culture

Day 5-8: Selection or Analysis

Mark Done

- **For fluorescent markers:** Sort by FACS on Day 7-8
- **For selection markers:** Begin antibiotic selection on Day 3-5
- Common antibiotics: Puromycin (1-5 µg/mL), Blasticidin (5-10 µg/mL), G418 (400-800 µg/mL)
- Maintain selection pressure for 5-7 days
- Untransduced control cells should die within 3-5 days

Alternative: Plate-Based Infection (No Spinoculation)

- Seed cells in 6-well plate at desired density
- Add virus + polybrene (8 µg/mL) directly to wells
- Incubate 24 hours, then change medium
- Lower efficiency than spinoculation but simpler

Troubleshooting

Problem	Possible Cause	Solution
Low virus titer	293T cells over/under confluent Old plasmids Transfection inefficient	Optimize cell density (60-70% at transfection) Use fresh, high-quality plasmids Optimize DNA:reagent ratio Verify plasmid sequences
293T cells died after transfection	Toxic transfection reagent Media change too late	Change media 4-8 hours post-transfection Reduce transfection reagent amount Use healthier passage of 293T
Low infection efficiency	Low virus titer Target cells resistant No polybrene	Use concentrated virus Increase virus volume (MOI) Add polybrene (8 µg/mL) Use spinoculation Optimize for cell type
High cytotoxicity during infection	Too much polybrene High MOI	Reduce polybrene to 4-6 µg/mL Reduce virus amount

Problem	Possible Cause	Solution
		Change media 4 hours post-infection
Variable expression across cells	Multiple integration events Low MOI	Use lower MOI (0.3-1.0) FACS sort for uniform expression Clone single cells if needed
Virus titer decreased after freezing	Freeze-thaw damage Ice crystal formation	Snap freeze in liquid nitrogen or dry ice/ethanol Store at -80°C (not -20°C) Aliquot to avoid freeze-thaw cycles Thaw rapidly at 37°C

Tips for Success

- Use low-passage 293T cells (<P20)
- Verify plasmid integrity before transfection
- Collect virus 48-72h post-transfection (peak titer)
- Filter virus immediately after collection
- Aliquot virus to minimize freeze-thaw
- Optimize MOI for each cell type (typically 0.5-5)
- Include untransduced controls for selection

Safety Reminders

- All work in BSL-2 biosafety cabinet
- Decontaminate with 10% bleach (30 min contact)
- Autoclave all contaminated materials
- Never store lentivirus in shared -20°C freezers
- Label all tubes clearly: "LENTIVIRUS - BSL-2"
- Report spills to EHS immediately