# The RNAi Consortium (TRC)
# Broad Institute

**TRC Laboratory Protocols**
Protocol Title: **Lentivirus production of shRNA or ORF-pLX clones**
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## Brief Description:

This protocol describes production of lentivirus stocks from pLKO (shRNA) or pLEX (ORF) plasmids in 10 cm plates, in 6-well plates, and in high-throughput format (96-well plates).

## Table of Contents:



## Workflow Timeline:

In general, lentiviral production consists of the following steps with a typical timeline as noted:

| | |
|---|---|
| Day 0 | Seed 293T packaging cells |
| Day 1 (pm) | Transfect packaging cells with 3 lentivirus plasmids (pLKO or pLEX, packaging plasmid, envelope plasmid) |
| Day 2 (am) | *18 hours post-transfection*: Remove media; replace with fresh high-BSA or high-serum media |
| Day 3 (am) | *24 hours after media change*: Harvest virus; replace with fresh high-BSA or high-serum media |
| Day 4 (am) | *24 hours after harvest 1:* Harvest virus; discard packaging cells |

These procedures should be carried out in accordance with biosafety requirements of the host institution.

## Part 1: Cell Maintenance

We have observed that virus production yields can be significantly affected by the history of culturing conditions of the cells used for packaging of virus. We use 293T cells which were empirically selected from among cells obtained from several sources to be particularly adherent in the microtiter plates used for viral packaging.

For cell maintenance, we recommend:

- Split cells 3 times a week. For T75, plate 1E6 (Monday), 1E6 (Wednesday) and 8E5 (Friday) cells per flask with a total volume of 15 mL. For T175, plate 2.5E6 (Monday), 2.5E6 (Wednesday) and 2E6 (Friday) cells per flask with a total volume of 35 mL.
- Do not add Pen/Strep in either maintenance media or the media for seeding cells.
- Keep cell passage below 15.

## Part 2: Lentiviral Production in 10 cm plates

### I. Materials

Transfection-quality plasmid DNA for:
- pLKO/pLEX vector (TRC library plasmid – see DNA prep protocol)
- 2nd generation packaging plasmid containing *gag*, *pol* and *rev* genes (e.g. pCMV-dR8.91 or pCMV-dR8.74psPAX2)*
- envelope plasmid (e.g. VSV-G expressing plasmid, pMD2.G)*

** recommended: use endotoxin-free plasmid isolation kits (Qiagen)*

TransIT-LT1 transfection reagent (Mirus Bio, MIR 2300/5/6)
*alternative*: FuGENE 6 (Roche, #1 814 443 or #1 988 387)
OPTI-MEM serum-free media (Invitrogen, #31985-070)
293T packaging cells
Cell maintenance/seeding media: Antibiotic-free 293T growth media (DMEM + ~10% iFBS + 0.1x Pen/Strep)
500 mL DMEM (Dulbecco's Modification of Eagle's Medium; e.g. Mediatech #10-013-CV)
50 mL iFBS (heat-inactivated Fetal Bovine Serum; e.g. HyClone #SH30071.03)
Viral harvest media: High-BSA 293T growth media (DMEM + ~10% iFBS + ~1g/100mL BSA + 1x Pen/Strep)
500 mL DMEM (Dulbecco's Modification of Eagle's Medium; e.g. Mediatech #10-013-CV)
50 mL iFBS (heat-inactivated Fetal Bovine Serum; e.g. HyClone # SH30071.03)
64 mL 10g/100mL BSA stock (microbiology-grade Bovine Serum Albumin; VWR #14230-738)
5 mL 100x Pen/Strep (10,000 IU/mL penicillin, 10,000 µg/mL streptomycin; e.g. Mediatech #30-002-CI)
*alternative viral harvest media:* High-serum 293T growth media (DMEM + 30% iFBS + 1x Pen/Strep)
500 mL DMEM (Dulbecco's Modification of Eagle's Medium; e.g. Mediatech #10-013-CV)
200 mL iFBS (heat-inactivated Fetal Bovine Serum; e.g. HyClone # SH30071.03)
5 mL 100x Pen/Strep (10,000 IU/mL penicillin, 10,000 µg/mL streptomycin; e.g. Mediatech #30-002-CI)
10 cm tissue culture plates
Polypropylene storage tubes

### II. Instructions

1. Seed 293T packaging cells at $3.8x10^5$ cells/mL (10 mL per plate) in antibiotic-free growth media (DMEM + 10% iFBS) in 10 cm tissue culture plates.

2. Incubate cells for 24 hours (37 °C, 5% $CO_2$), or until the following afternoon. After ~24 hours, the cells should be 70-80% confluent.

3. Transfect packaging cells:
   a. Prepare a mixture of the 3 transfection plasmids:

| Reagent | per 10 cm plate |
|---|---|
| packaging plasmid (e.g. pCMV-dR8.91 or pCMV-R8.74psPAX2) | 9 µg |
| envelope plasmid (e.g. VSV-G/pMD2.G) | 0.9 µg |
| pLKO or pLEX vector | 9 µg |

| | |
|---|---|
| OPTI-MEM to total volume | 225 µL |

b. Dilute TransIT-LT1 transfection reagent in OPTI-MEM. Add the TransIT-LT1 reagent dropwise and mix by swirling the tip or gently flicking the tube (do not mix by pipetting or vortexing). Incubate 5 minutes at room temperature.

| Reagent | per 10 cm plate |
|---|---|
| TransIT-LT1 | 54 µL |
| OPTI-MEM to total volume | 90 µL |

c. Add the diluted TransIT-LT1 reagent to the 3 plasmid mix dropwise and mix by swirling the tip or gently flicking the tube.

d. Incubate the transfection mix for 30 minutes at room temperature.

e. Carefully transfer the transfection mix to the packaging cells (in antibiotic-free growth media). The packaging cells can be sensitive to perturbation - take care not to dislodge the cells from the plate.

4. Incubate cells for 18 hours (37 °C, 5% $CO_2$), or until the following morning.

5. Change media to remove the transfection reagent and replace with 15 mL high-BSA growth media or high serum growth media for viral harvests.

6. Incubate cells for 24 hours (37 °C, 5% $CO_2$).

7. Harvest 15 mL media containing lentivirus. Transfer the harvested media to a polypropylene storage tube. Add 15 mL high-BSA growth media or high serum growth media to the cells.

8. Repeat viral harvesting after 24 hours. After the final harvest, discard the packaging cells. The viral harvests may be pooled as desired.

9. Spin the media containing virus at 1250 rpm for 5 minutes to pellet any packaging cells that were collected during harvesting. Transfer the supernatant to a sterile polypropylene storage tube.

10. Virus may be stored at 4 °C for short periods (hours to days), but should be frozen at -20 °C or -80 °C for long-term storage. To reduce the number of freeze/thaw cycles, aliquot large-scale virus preps to smaller storage tubes prior to long-term storage.

## Part 2: Lentiviral Production in 6-well Plates

*(**Note**: While this protocol works, we haven't fully optimized it to maximize the viral titer, as 6-well prep is not a major workflow for us.)*

### I. Materials

Transfection-quality plasmid DNA for:

- pLKO or pLEX vector (TRC library plasmid – see DNA prep protocol)
- 2nd generation packaging plasmid containing *gag*, *pol* and *rev* genes (e.g. pCMV-dR8.91 or pCMV-dR8.74psPAX2)
- envelope plasmid (e.g. VSV-G expressing plasmid, pMD2.G)

TransIT-LT1 transfection reagent (Mirus Bio, MIR 2300/5/6)
*alternative*: FuGENE 6 (Roche, #1 814 443 or #1 988 387)
OPTI-MEM serum-free media (Invitrogen, #31985-070)
293T packaging cells
Cell seeding media: Antibiotic-free 293T growth media (DMEM + ~10% iFBS)
500 mL DMEM (Dulbecco's Modification of Eagle's Medium; e.g. Mediatech #10-013-CV)
50 mL iFBS (heat-inactivated Fetal Bovine Serum; e.g. HyClone #SH30071.03)
Viral harvest media: High-BSA 293T growth media (DMEM + ~10% iFBS + ~1g/100mL BSA + 1x Pen/Strep)
500 mL DMEM (Dulbecco's Modification of Eagle's Medium; e.g. Mediatech #10-013-CV)
50 mL iFBS (heat-inactivated Fetal Bovine Serum; e.g. HyClone # SH30071.03)
64 mL 10g/100mL BSA stock (microbiology-grade Bovine Serum Albumin; VWR #14230-738)
5 mL 100x Pen/Strep (10,000 IU/mL penicillin, 10,000 µg/mL streptomycin; e.g. Mediatech #30-002-CI)
*alternative viral harvest media:* High-serum 293T growth media (DMEM + 30% iFBS + 1x Pen/Strep)
500 mL DMEM (Dulbecco's Modification of Eagle's Medium; e.g. Mediatech #10-013-CV)
200 mL iFBS (heat-inactivated Fetal Bovine Serum; e.g. HyClone # SH30071.03)
5 mL 100x Pen/Strep (10,000 IU/mL penicillin, 10,000 µg/mL streptomycin; e.g. Mediatech #30-002-CI)
6-well tissue culture plates (e.g. Corning/Costar #3506)
Polypropylene storage tubes

**II. Instructions**

1. Seed 293T packaging cells at $2.2x10^5$ cells/mL (2.5 mL per well) in antibiotic-free growth media (DMEM + 10% iFBS) in 6-well tissue culture plates.

2. Incubate cells for 24 hours (37 °C, 5% $CO_2$), or until the following afternoon. After ~24 hours, the cells should be 70-80% confluent.

3. Transfect packaging cells:
   a. Prepare a mixture of the 3 transfection plasmids:

| Reagent | per well |
|---|---|
| packaging plasmid (e.g. pCMV-dR8.91 or pCMV-R8.74psPAX2) | 500 ng |
| envelope plasmid (e.g. VSV-G/pMD2.G) | 50 ng |
| pLKO or pLEX vector | 500 ng |
| OPTI-MEM to total volume | 37.5 µL |

   b. Dilute TransIT-LT1 transfection reagent in OPTI-MEM. Add the TransIT-LT1 reagent dropwise and mix by swirling the tip or if mix volume permits gently flicking the tube (do not mix by pipetting or vortexing). Incubate 5 minutes at room temperature.

| Reagent | per well |
|---|---|
| TransIT-LT1 | 3 µL |
| OPTI-MEM to total volume | 15 µL |

c. Add the diluted TransIT-LT1 reagent to the 3 plasmid mix dropwise and mix by swirling the tip or gently flicking the tube.

d. Incubate the transfection mix for 30 minutes at room temperature.

e. Carefully transfer the transfection mix to the packaging cells (in antibiotic-free growth media). The packaging cells can be sensitive to perturbation - take care not to dislodge the cells from the plate.

4. Incubate cells for 18 hours (37 °C, 5% $CO_2$), or until the following morning.

5. Change media to remove the transfection reagent and replace with 2.5 mL high-BSA growth media or high serum growth media for viral harvests.

6. Incubate cells for 24 hours (37 °C, 5% $CO_2$).

7. Harvest 2.5 mL media containing lentivirus. Transfer the harvested media to a polypropylene storage tube. Add 2.5 mL high-BSA growth media or high serum growth media to the cells.

8. Repeat viral harvesting after 24 hours. After the final harvest, discard the packaging cells. The viral harvests may be pooled as desired.

9. Spin the media containing virus at 1250 rpm for 5 minutes to pellet any packaging cells that were collected during harvesting. Transfer the supernatant to a sterile polypropylene storage tube.

10. Virus may be stored at 4 °C for short periods (hours to days), but should be frozen at -20 °C or -80 °C for long-term storage. To reduce the number of freeze/thaw cycles, aliquot large-scale virus preps to smaller storage tubes prior to long-term storage.

## Part 3: High-Throughput Lentiviral Production (96 well plates)

### I. Materials

Transfection-quality plasmid DNA for:
- pLKO/pLEX (TRC library plasmid – see DNA prep protocol)
- 2nd generation packaging plasmid containing *gag*, *pol* and *rev* genes (e.g. pCMV-dR8.91 or pCMV-dR8.74psPAX2)
- envelope plasmid (e.g. VSV-G expressing plasmid, pMD2.G)

TransIT-LT1 transfection reagent (Mirus Bio, MIR 2300/5/6)
*alternative*: FuGENE 6 (Roche, #1 814 443 or #1 988 387)
OPTI-MEM serum-free media (Invitrogen, #31985-070)
293T packaging cells
Cell seeding media: Antibiotic-free 293T growth media (DMEM + ~10% iFBS)
500 mL DMEM (Dulbecco's Modification of Eagle's Medium; e.g. Mediatech #10-013-CV)
50 mL iFBS (heat-inactivated Fetal Bovine Serum; e.g. HyClone #SH30071.03)

Viral harvest media: High-BSA 293T growth media (DMEM + ~10% iFBS + ~1g/100mL BSA + 1x Pen/Strep)
- 500 mL DMEM (Dulbecco's Modification of Eagle's Medium; e.g. Mediatech #10-013-CV)
- 50 mL iFBS (heat-inactivated Fetal Bovine Serum; e.g. HyClone # SH30071.03)
- 64 mL 10g/100mL BSA stock (microbiology-grade Bovine Serum Albumin; VWR #14230-738)
- 5 mL 100x Pen/Strep (10,000 IU/mL penicillin, 10,000 µg/mL streptomycin; e.g. Mediatech #30-002-CI)

*alternative viral harvest media:* High-serum 293T growth media (DMEM + 30% iFBS + 1x Pen/Strep)
- 500 mL DMEM (Dulbecco's Modification of Eagle's Medium; e.g. Mediatech #10-013-CV)
- 200 mL iFBS (heat-inactivated Fetal Bovine Serum; e.g. HyClone # SH30071.03)
- 5 mL 100x Pen/Strep (10,000 IU/mL penicillin, 10,000 µg/mL streptomycin; e.g. Mediatech #30-002-CI)

96-well tissue culture plates (e.g. Corning/Costar #3628)
96-well polypropylene storage plates (e.g. Corning/Costar #3357)

## II. Instructions

1. Seed 293T packaging cells at $2.2x10^5$ cell/mL (100 µL per well) in antibiotic-free growth media (DMEM + 10% iFBS) in 96-well tissue culture plates. Allow seeded plates to sit undisturbed at room temperature for at least 1 hour before transferring to a tissue culture incubator overnight.
   *Note: allowing cells to settle at room temperature can reduce well-to-well variability and edge effects in microtiter plates.*

2. Incubate cells for 24 hours (37 °C, 5% $CO_2$), or until the following afternoon. After ~24 hours, the cells should be 70-80% confluent.

3. Transfect packaging cells:

   a. Prior to virus prep, pLKO or pLEX plasmids are normalized to 15 ng/µl. Transfer 100 ng (6.7 µl) of such normalized plasmids to a sterile 96-well polypropylene storage plate pre-loaded with 10 µl sterile $H_2O$ (to facilitate liquid transfer by robots).

   b. Prepare a mixture of the packaging and VSV-G envelope plasmids:

| Reagent | per well* |
|---|---|
| packaging plasmid (e.g. pCMV-dR8.91 or pCMV-dR8.74psPAX2) | 100 ng |
| envelope plasmid (e.g. VSV-G/pMD2.G) | 10 ng |
| OPTI-MEM to total volume | 10 µL** |

   * Volumes do not account for excess dead volume required for multichannel pipette reservoirs or liquid handling robots.
   ** The volume of OPTI-MEM per well can be adjusted for optimal handling and automated setup.

   c. Dispense the packaging plasmid mix (10 µL per well) into the sterile 96-well polypropylene storage plate containing shRNA or ORF-pLX plasmids.

d. Dilute TransIT-LT1 transfection reagent in OPTI-MEM: Add the TransIT-LT1 reagent dropwise and mix by swirling the tip or gently flicking the tube (do not mix by pipetting or vortexing). Incubate 5 minutes at room temperature.

| Reagent | per well* |
|---|---|
| TransIT-LT1 | 0.6 µL |
| OPTI-MEM to total volume | 10 µL** |

* Volumes do not account for excess dead volume required for multichannel pipette reservoirs or liquid handling robots.

** The volume of OPTI-MEM per well can be adjusted for optimal handling and automated setup.

e. Dispense the diluted TransIT-LT1 (10 µL per well) to the 3-plasmid mix plate. Mix gently by pipetting.

f. Incubate the transfection plate for 30 minutes at room temperature.

g. Carefully transfer the transfection mix to the packaging cells (in antibiotic-free growth media). The packaging cells can be sensitive to perturbation - take care not to dislodge the cells from the plate.

4. Incubate cells for 18 hours (37 °C, 5% $CO_2$), or until the following morning.

5. Change media to remove the transfection reagent and replace with 170 µL high-BSA growth media or high serum growth media for viral harvests.
*Note: Lentivirus will start to appear in the media supernatant ~22 hours post-transfection.*

6. Incubate cells for 24 hours (37 °C, 5% $CO_2$), or until the following morning.

7. Harvest 150 µL media containing lentivirus and transfer to a 96-well polypropylene storage plate. Replace with 170 µL high-BSA growth media or high serum growth media for viral harvests.
*Note: The first harvest may be stored at 4 °C for 24 hours if the harvests will be pooled.*

8. Incubate cells for 24 hours (37 °C, 5% $CO_2$), or until the following morning.

9. Harvest $150^+$ µL media containing lentivirus and transfer to a 96-well polypropylene storage plate. Discard the packaging cells.

10. If desired, pool viral harvests and/or rearray to 96-well or 384-well plates. Virus may be stored at 4 °C for short periods (hours to days), but should be frozen at -20 °C or -80 °C for long term storage.

**Version Notes:**

4/10/06

**Transfection reagent:** TransIT-LT1 (MirusBio) has the same performance as FuGene 6 (Roche) in our comparison tests. Either transfection reagent may be used for virus production. As of this version date, TransIT-LT1 has a lower list price.

**Harvest volume and timeline:** In previous HT protocols, we recommended 3 media harvests (100 µL each) at ~36, ~48, and ~60 hours post-transfection. We recover the equivalent (or higher) virus yield with 2 media harvests (150 µL each) at ~36 and ~60 hours post-transfection.

**High-serum growth media:** We have found that increasing the amount of serum to 30% in the virus production media improves virus yield by ~2-fold.

1/18/07
**High-BSA growth media for viral harvests**: We have found that viral harvest growth media containing 10% serum + 1.1g/100mL supplemental BSA is equivalent to viral harvest media containing 30% serum – both produce viral stocks with similar high titer. The BSA-supplemented media is more cost effective, easier to mix in standard 500mL media bottles, and may be preferred when transfecting cells that are sensitive to serum.

**Packaging plasmid:** the pCMV-dR8.91and pCMV-dR8.74psPAX2 packaging plasmids are equivalent; both produce equivalent high-titer viral stocks. pCMV-dR8.74psPAX2 ("psPax2") and the envelope plasmid pMD2.G are available from Addgene (www.addgene.org): psPax2 = plasmid #12260, pMD2.G plasmid #12259.

8/9/10
**Added 6 well plate version of lentivirus production protocol**

10/20/12
**Cell maintenance notes added:** 3x passages/week and removing pen/strep in maintenance/seeding media help produce high titer virus (>2E8 IU/mL).

**10 CM dish prep protocol updated:** We optimized cell number and pDNA quantity for this workflow. By increasing cell number from 2.2E6 to 3.8E6/well and 3-pDNA from 3:3:0.3µg to 9:9:0.9µg, we saw 2x fold increase in virus titer (to ~1E8 IU/mL).