

The RNAi Consortium (TRC)

Broad Institute

TRC Laboratory Protocols

Protocol Title: **Lentivirus production of shRNA, CRISPR, or ORF-pLX clones in 10 cm dishes or 6-well plates**

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Brief Description:

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

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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, pXPR or pLEX, packaging plasmid, envelope plasmid)
Day 2 (am)	<i>18 hours post-transfection</i> : Remove media; replace with fresh high-BSA or high-serum media
Day 3 (am)	<i>24 hours after media change</i> : Harvest virus; replace with fresh high-BSA or high-serum media
Day 4 (am)	<i>24 hours after harvest 1</i> : 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.

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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 3.8×10^5 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% CO₂), 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

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- 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 μ L
OPTI-MEM to total volume	90 μ L

- 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% CO₂), 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% CO₂).
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)

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- 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 2.2×10^5 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% CO₂), 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

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- 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% CO₂), 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% CO₂).
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.

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%

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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-dR8.91 and 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).

6/3/15

Separated high throughput 96-w viral prep protocol from the low throughput methods (i.e. preps using 10 cm dishes and 6-well plates)