TRC Laboratory Protocols
Protocol Title: **High throughput lentivirus production of shRNA, CRISPR, or ORF-pLX clones**
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Brief Description:

This protocol describes the production of lentivirus stocks from pLKO (shRNA), pXPR (CRISPR), or pLEX (ORF) plasmids in high-throughput format (96-well plates).

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Workflow Timeline:

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

2 harvests protocol:

Day 0 (8 am)	Seed 293T packaging cells
Day 1 (7 am)	Transfect packaging cells with 3 lentivirus plasmids (pLKO, pXPR, or pLEX, packaging plasmid, envelope plasmid)
Day 1 (1 pm)	*6 hours post-transfection*: Remove media; replace with fresh high-BSA or high-serum media
Day 2 (3 pm)	*32 hours post-transfection*: Harvest virus; replace with fresh high-BSA or high-serum media
Day 3 (3 pm)	*24 hours after harvest 1 (i.e. 56 hours post-transfection):* Harvest virus; discard packaging cells

Single harvest protocol (use if you need the highest possible titer):

Day 0 (8 am)	Seed 293T packaging cells
Day 1 (7 am)	Transfect packaging cells with 3 lentivirus plasmids (pLKO, pXPR, or pLEX, packaging plasmid, envelope plasmid)
Day 1 (1 pm)	*6 hours post-transfection*: Remove media; replace with fresh high-BSA or high-serum media
Day 3 (8 am)	*48 hours post-transfection:* Harvest virus; discard packaging cells

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

Part 1: Cell Maintenance

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

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: 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). 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, CRISPR, 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 6 hours (37 °C, 5% CO_2).

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 ~18 hours post-transfection.

   Note: For most vectors, our 2-harvest virus preps yield very high titer virus. Only in rare cases where some vectors have difficulties packaging, we found a single harvest at 48 hours post-transfection gives the highest possible titer.

   For 2-harvest protocol, do step 6 and step 7. For single-harvest protocol, do step 8 instead.
6. (For 2-harvest protocol) Incubate cells for 26 hours (37 °C, 5% CO_2). 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.

7. (For 2-harvest protocol) Incubate cells for 24 hours (37 °C, 5% CO_2). Harvest 150^+ µL media containing lentivirus and transfer to a 96-well polypropylene storage plate. Discard the packaging cells.

8. (For single-harvest protocol) Incubate cells for 42 hours (37 °C, 5% CO_2). Harvest 150^+ µL media containing lentivirus and transfer to a 96-well polypropylene storage plate. Discard the packaging cells.

9. 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).

06/03/15
Lab event timeline re-optimized: 96-w plate based prep was optimized with new timelines including 6 hour post-transfection medial change, and new best virus harvest timepoints depending on options of 2-, or single-harvest.

Remove protocols of 10 cm dish prep or 6-well plate prep. These 2 protocols are residing in a separate protocol titled 'Lentivirus production of shRNA, CRISPR, or ORF-pLX clones in 10 cm dishes or 6-well plates.'