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Protocol: 96-well format lentivirus production of shRNA, CRISPR, or ORF-pLX clones

Brief Description:

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

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Workflow Timeline:

Below is a typical timeline for lentiviral production. For most vectors, our double-harvest virus preps yield very high titer virus. Only in rare cases where some vectors have difficulties packaging (for example pLX317 or pXPR_023), we found a single harvest at 48 hours post-transfection gives the highest possible titer.

Single harvest 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 3 (8 am)	*48 hours post-transfection:* Harvest virus; discard packaging cells

Double harvest 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

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 splitting cells 3 times a week. In a T-75 flask (15 ml of media), plate 1E6 cells (Monday), 1E6 cells (Wednesday) and 8E5 cells (Friday) per flask. In a T175 (35 ml of media), plate 2.5E6 (Monday), 2.5E6 (Wednesday) and 2E6 (Friday) cells per flask.
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 96-well plate format

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 Addgene Cat# 12260)
- envelope plasmid (e.g. VSV-G expressing plasmid, pMD2.G, Addgene Cat# 12259)

TransIT-LT1 transfection reagent (Mirus Bio, MIR 2300/5/6) or 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)
Either viral harvest media can be used, the one containing BSA is more cost-effective since it uses less FBS
-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 2.2×10^5 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.

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 the plasmids to a sterile 96-well polypropylene storage plate pre-loaded with 10 µl sterile H2O (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 of viral harvest media selected (either high-BSA 10% serum or 30% serum).
   Lentivirus will start to appear in the media supernatant ~18 hours post-transfection.

For double-harvest protocol, go to steps 6-7. For single-harvest protocol, go to step 8-9 instead.

6. (For double-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 viral harvest media selected (either high-BSA 10% serum or 30% serum).
   You may store the first harvest at 4 °C for 24 hours while waiting for the second harvest and then pool.
7. (For double-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.