Lentiviral transduction of cells

The lentiviral system we have in the Springer Lab is using vectors that we got from Florian Winau's Lab. It was developed at the Broad Institute and was used in the human ORFeome project. It is a second-generation system using three vectors for virus production: psPAX2, pCMV-VSVG, and pLX302 containing the gene of interest. Cloning of the pLX302 lentiviral construct uses Invitrogen's Gateway system. Detailed information on those components can be found here:

- **pLX302 vector**: http://www.addgene.org/25896/
- **pCMV-VSVG vector**: http://www.addgene.org/8454/
- **psPAX2 vector**: http://www.addgene.org/12260/
- **pDONR223 vector**: https://www.lablife.org/g?a=seqa&id=vdb_g2.PCivHaM6lS6z72tbBQRTiEIE82Q-_sequence_22b444e627165e48341aee2425754bfef459c3f2_10
- **hORFeome methods paper**: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3234135/?tool=pubmed
- **hORFeome @ The Broad**: http://www.broadinstitute.org/rnai/orfs
- **Gateway cloning system (Invitrogen)**: http://tools.invitrogen.com/content/sfs/manuals/gateway_clonaseii_man.pdf

Part I: Generation of ENTRY clone

First, you need to clone your gene of interest into a vector that is compatible with Gateway cloning. Empty Gateway plasmids to start with are called pDONR vectors. We are using the pDONR223 plasmid. The so-called BP reaction allows you to transfer your insert of interest into the pDONR vector using recombinases, thereby generating a pENTRY vector that can be used for Gateway cloning. The empty pDONR223 has spectinomycin and chloramphenicol resistance cassettes (see vector map). The chloramphenicol resistance (CAT) plus a bacteriotoxic ccdB gene are flanked by the attP recombination sites and get both replaced by your gene of interest in the BP reaction. Since DH5α are extremely sensitive to ccdB, the empty pDONR223 (as well as the empty pLX302) have to be propagated in the ccdB Survival 2 T1R strain (Invitrogen) in presence of spectinomycin plus chloramphenicol. After the BP reaction, DH5α are transformed, and only clones in which the ccdB cassette has been replaced by your gene will grow in LB-Spectinomycin.

Material:

- pDONR223 plasmid
- Primers with attB flanking sequences (see Gateway manual)
- DNA polymerase for PCR
- Gateway BP Clonase II Enzyme Mix (Invitrogen #11789-020)
- Proteinase K (contained in Clonase Mix)

- Subcloning-efficiency DH5α
- SOC medium
- LB-spectinomycin plates (50 µg/ml)
- Spectinomycin
- 50% glycerol for freezing bacterial stocks
- Miniprep kit
- Sequencing primers (M13F, M13R, construct-specific primers)

1. PCR amplify your gene of interest using primers that have flanking attB1 (Fwd primer) and attB2 (Rev primer) recombination sites (see Gateway manual). Don't forget to include a Kozak sequence. The pLX302 vector has a C-terminal V5 tag followed by a stop codon immediately after the Gateway attP2 sequence. Make sure that your construct is in-frame if you would like to use that V5 tag, or include a stop codon before the attB2 reverse primer.
   attB1: 5'-GGG G**AC AAG TTT GTA CAA AAA AGC AGG CT(Kozak-ATG-gene)**-3'
   attB2: 5'-GGG G**AC CAC TTT GTA CAA GAA AGC TGG GT(±STOP-gene)**-3'
2. Confirm correct size of PCR products and gel-purify them.
3. Perform BP reaction:
   25 fmol pDONR223 (=75 ng)
   25 fmol insert carrying flanking attB sites
   1 µl BP Clonase II
   to 5 µl with TE buffer, pH 8.0

   Incubate at room temperature overnight
   Controls: pDONR/no enzyme; pDONR+PCR product/no enzyme

4. Add 0.5 µl of proteinase K (2 µg/µl; provided with enzyme), incubate @ 37 °C for 10 min
5. Transform 20 µl DH5α with entire reaction, add 480 µl SOC. Incubate for 1 h at 37 °C
6. Plate 50 µl on spectinomycin (50 µg/ml) plates. Incubate overnight at 37 °C.
7. Control plates should have very few, if any colonies. BP reaction plates should have >100 colonies.
8. Grow up pENTRY clones in LB/spectinomycin, freeze glycerol stocks, and check sequence. M13F and M13R primers can be used.

Part II: Generation of pLX302 lentivirus destination vector

Here, your gene of interest is shuttled from the pENTRY vector to the Gateway Destination vector, in our case pLX302. This step is called LR reaction and is catalyzed by the LR Clonase Enzyme Mix. As for other empty Gateway plasmids, keep in mind to only propagate the empty pLX302 in the ccdB Survival 2 T1R E. coli strain. Culture of lentiviral plasmids is tricky, because spontaneous recombination can occur and alter the plasmid. Therefore, those vectors are cultured at 30 °C for 18-24 h rather than 37 °C.

Material:

- pLX302 (culture in ccdB Survival 2 T1R; LB+carbenicillin+chloramphenicol; 30°C for 24 h)
- pENTRY vectors carrying gene of interest
- Gateway® LR Clonase® II enzyme mix (Invitrogen #11791-020)
- Proteinase K (2 µg/µl), provided with enzyme mix
- Subcloning-efficiency DH5α
- Carbenicillin
- 50% glycerol for freezing bacterial stocks
- Miniprep or Midiprep kit
- BsrGI enzyme

1. Perform LR reaction:
   75 ng pLX302
   75 ng pENTRY vector
   1 µl LR Clonase II enzyme mix
   to 5 µl with TE buffer, pH 8.0

   Room temperature for 24 h; controls w/o enzyme

2. Add 0.5 µl proteinase K (2 µg/µl), 37 °C for 10 min
3. Transform 20 µl DH5α with entire reaction volume. Add 480 µl SOC, grow 1 h @ 30 °C
4. Plate 50 µl and 450 µl on LB-Amp or LB-Carb plates at 30 °C for 18-24 h. Control plates should not show colonies.
5. Pick clones and grow in LB-Carb for 18-24 h at 30 °C, freeze glycerol stocks, purify plasmids.
6. Check for presence of insert in plasmids using BsrGI digestion at 55 °C. Empty non-recombined pLX302 gives 402 bp, 1283 bp, and 7888 bp fragments.

Part III: Packaging of lentivirus

Here, the infectious lentivirus particles are generated and packed using the 293T cell line. All the work has to be done under BL2 safety standards. Always have a beaker of bleach in the hood to immediately inactivate liquid waste. We use a second-generation system with co-transfection of three plasmids: (1) the lentiviral vector pLX302 that contains your gene-of-interest and LTRs, (2) the pCMV-VSVG plasmid for pseudo-typing with VSVG, allowing the lentivirus to infect a broad range of cells, and (3) the psPAX2 plasmid with *gag*, *pol*, and *rev* genes. As a control, we have the FUGW plasmid that is used instead of pLX302 constructs and generates an EGFP lentivirus. If possible, use endotoxin-free plasmid preps.

Material:

- psPAX2
- pCMV-VSVG
- pLX302 constructs
- 0.001% Poly-L-lysine in PBS
- PBS
- 293T cells
- 293T medium (DMEM+10% FBS+2 mM Gln+NEAA; no antibiotics)
- 6-well plates
- Lipofectamine 2000
- Opti-MEM I
- FUGW positive control (EGFP)
- Virus harvest medium (DMEM+10% FBS+6 mM Gln+NEAA+1.1 g/L BSA+1 mM sodium pyruvate+antibiotics). Get 1.1 g/L BSA by adding 32 ml of 20 g/100 ml BSA stock per 500 (+50) ml bottle.

1. Coat wells with 0.001% poly-L-lysine in PBS for 1 h at 37 °C. Wash twice with PBS and air-dry in biosafety cabinet. This will help the 293T cells to stay attached over the virus production period.
2. Resuspend 293T cells in antibiotic-free medium. Plate in 6-well plates, 2.5 ml/well @ 2.2×10^5 cells/ml and culture for 24 h under 10% CO_2.
3. Cells should be 80-95% confluent. Prepare transfection mix: Per well, use 500 ng pLX302 construct, 50 ng pCMV-VSVG, and 450 ng psPAX2. Dilute plasmids in 30 µl Opti-MEM I. Dilute Lipofectamine 2000, 2 µg in 30 µl Opti-MEM I per well. Let hydrate for 5 min. Combine plasmids and LF2000. Incubate for 30 min at RT, then add to cells.
4. Incubate cells at 37 °C under 10% CO_2 for 18 h.
5. Replace medium by 2.5 ml/well virus harvest medium. Incubate at 37 °C under 5% CO_2 for 24 h. Already treat this liquid waste as potentially virus-containing. Typically, infectious particles start to be secreted 30-36 h post-transfection.
6. Collect virus-containing supernatant and replace with fresh virus harvest medium, 2.5 ml/well. Store supernatant at 4 °C.

7. After another 24 h, collect second batch of supernatant and pool with first one. If necessary, virus-containing supernatant can be buffered with 25 mM HEPES pH 7.4. Pass through 0.45 µm filter to remove any 293T cells. If necessary, spin supernatant first. Do not filter through 0.22 µm filter, because virus could be sheared and inactivated.
8. Use virus immediately for transduction or freeze aliquots at -80 °C. Avoid repeated freeze-thawing.

Part IV: Lentiviral transduction

The protocol in this part is based on successful experiments with B lymphocytes. Conditions for other cell types need to be optimized. Lentiviruses can always be tested using 293T cells as targets; they are transduced very efficiently. If titer is too low, virus can be concentrated by various methods, including ultracentrifugation. See literature for that.

Material:

- Filtered lentivirus supernatant
- Target cells
- Polybrene, 10 mg/ml, sterile filtered
- 24-well plate
- Medium for target cells
- Puromycin

1. Count target cells and spin down aliquots of $1x10^6$ cells.
2. Resuspend cells in 500 µl lentivirus supernatant and transfer to 24-well plate.
3. Add polybrene to a final concentration of 8 µg/ml (or optimized concentration). Mix well.
4. Centrifuge plate @ 2,000 rpm for 90 min at room temperature or 37 °C, if possible. Polybrene aggregates virus particles and allows sedimentation/enrichment at low speed.
5. Add another 500 µl fresh medium to cells and incubate for 24-48 h.
6. Replace medium with 1 ml selection medium containing puromycin (if pLX302 was used for virus packaging). Puromycin needs to be titrated with nontransfected cells. Typical concentrations range from 0.1-10 µg/ml. Protein expression can be monitored by flow cytometry at this point for cells transduced with FUGW control virus or a lentivirus encoding a cell surface receptor.
7. Continue selection process until all nontransduced control cells are dead.