Lentivirus production:

IMPORTANT: The biosafety office at your institution must be notified prior to use of this system for permission and for further institution-specific instructions. BL2 conditions should be used at all times when handling the virus. All decontamination steps should be performed using 70% ethanol/1% SDS. Gloves should be worn at all times when handling lentiviral preparations, transfected cells or the combined transfection reagent. Just remember that although this virus has been significantly modified for biosafety, it derived from HIV and with a VSV pseudotype human cells can be infected even if they are not dividing. That said, the following modifications have been made to prevent viral replication.

1. **Packaging vector lacks both LTRs and has no viral packaging signal (ψ)**
2. **The following viral genes have been deleted from the packaging vector: env, tat, rev, vpr, vpu, vif and nef.**
3. **Rev is supplied in trans on a different vector (RSV-Rev).**
4. **The vector expressing the packaged viral genome has a self-inactivating LTR (TATA box deletion) and expresses no viral gene products.**
5. **Envelope, in this case VSVG, is expressed on a separate vector.**

For more information please refer to the following papers.

Packaging vectors pMDLg/pRRE, CMV-VSVG and RSV-Rev):

Dull et al., A Third-Generation Lentivirus Vector with a Conditional Packaging System. J. Virol. 1998 72(11): 8463-8472.

Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science. 1996 Apr 12;272(5259):263-7.

Self inactivating LTR:

Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM. Development of a self-inactivating lentivirus vector. J Virol. 1998 Oct;72(10):8150-7.

293 Transfection

1. Plate 12 x 10^6 293.T in 20 ml on a 15 cm^2 plate 24 hours before transfection. In general, two 15cm plates per virus. It is essential that the cells be well-maintained and of relatively low passage number.
2. Mix the following DNAs (made w/ Endo-free Qiagen Kits) in a FACS tube. The DNAs should be in Endo-free TE at a concentration of 0.5µg/µl.
   For 3 plasmid system:
   20 µg vector,
   10 µg VSVG
   15 µg Δ 8.9
   For 4 plasmid, system (recommended),
   20 µg vector,
   10 µg VSVG
   10 µg RSV-REV
   10 µg pMDL g/p RRE
3. Add 400 µl 1.25 M $CaCl_2$ and 1.5 ml H_20 and mix by tapping gently.

The following steps are done 1 plate at a time.

4. Add 2 ml of 2X HBS dropwise to DNA mixture while bubbling with a Pasteur pipette. When finished, continue to bubble for 12-15 seconds.
5. Take plate of 293T out of the incubator (plate remains in incubator for long as possible), and add transfection mixture dropwise all over the plate. Gently swirl plate from front to back, and return immediately to incubator.
6. 3.5 to 4 hours later, remove media, wash 2x with 10ml warm PBS, and add 20 ml warm D10 onto plate and place in incubator.
7. 36-48 hours after transfection, harvest viral supernatant and spin @ 2000 rpm, 7 min at 4°C in a 50ml tube.
8. Filter viral SN through .45 um filter. Add 35ml of filtered supernatant to an ultracentrifuge tube. Balance tubes with additional media. Cover tubes with small piece of parafilm. (It is useful to titer some of the leftover supernatant to determine if there is loss of virus during concentration.)
9. Spin tubes using a SW-28 rotor @ 25,000 rpm, 90 min, 4°C. Decant liquid and leave tube upside down on kimwipe for 10 min. Aspirate remaining media being careful not to touch bottom of tube.
10. Add 15µl cold PBS (for embryo infections, or any volume you wish) and leave tube at 4°C O/N with no shaking.
11. To resuspend, hold tube at angle and pipet fluid over pellet 20 times, being careful not to touch pellet with tip. It is expected that the pellet not be resuspended after this is complete. This pellet does not contain virus and can be discarded.
12. Aliquot or use virus. Virus should be aliquoted, flash-frozen in liquid nitrogen and stored at -80. There should be no change in titer with freezing concentrated virus. Avoid multiple freeze-thaws.