Working with pLX Gateway-compatible lentiviral vectors

The Gateway system represents an efficient method for shuttling expression clones between plasmids. This system relies on enzyme-mediated site-specific recombination between sequences present in Donor/Entry vectors (pDONR223 or 221 backbone) and Destination/Expression vectors. The creation of Entry vectors requires the BP Clonase enzyme and the transfer of ORFs from Entry vectors requires the LR Clonase enzyme. (Useful Tip: Once ORFs have been shuttled into Expression vectors using LR reactions, a BP reaction with pDONR-223 (or 221) can be used to reconstitute Entry vectors using the Expression clone as the ORF Entry vector). For more information on the Gateway system, please see www.invitrogen.com.

pLX Gateway-compatible lentiviral vectors encode several critical elements including the PGK promoter driving expression of drug resistance or selectable marker (e.g. blast, puro), the cPPT element which promotes high viral titers, the CMV promoter driving ORF expression, the Gateway cassette encoding the Chloromphenicol Resistance gene and the ccDB topoisomerase poison, the WPRE element for enhanced RNA stability and the pLKO vector backbone encoding Amp/Carb resistance.

The ccDB poison is toxic to DH5alpha competent bacteria which ensures that unrecombined Destination vectors cannot propagate after the LR reaction. Thus, like other Gateway-compatible expression vectors, prior to introduction of ORF sequences, the pLX vectors must be propagated in ccDB resistant bacteria (see below). Transform these bacteria with pLX vectors and plate on amp/carb O/N at 37deg. To propagate large vectors such as pLX vectors in these bacteria, bacterial colonies must be grown in liquid culture (~16hr) at 30 deg using non-rich media (LB). To minimize the chance for accidental recombination, do not overgrow cultures (< ~18hr).

1) Perform LR reactions as follows:
   100ng Entry vector
   100ng pLX Destination vector
   0.5ul-1ul LR clonase
   5-10ul total volume in H20 or TE
   Incubate 1hr to O/N at 25deg. LR efficiency will increase with overnight incubation. **note: be sure to perform negative control (Destination vector only)
2) Transform 2ul LR reaction into DH5alpha competent bacteria (DH5alpha are extremely ccDB sensitive, which is desired), plate on Amp/Carb plates. **note: do not use other types of competent cells, even if advertised as ccDB sensitive.
3) Confirm that background on negative control is extremely low (0-3 colonies). Successful LR reactions should have 100s to 1000s of colonies.
4) Inoculate 1 colony into rich media (TB), grow O/N at 37deg.
5) If desired, can confirm by sequencing using either pLX-ORFfwd or pLX-ORFrev primers, or by digesting with BsrG1 restriction enzyme which cuts in Gateway sequences which flank ORF. Typically we achieve >95% success when picking a single colony.

ORF-encoding pLX vector is now ready for creation of lentivirus. Protocols are identical to creating pLKO lentivirus. Typically more virus is required for ORF-encoding pLX vectors than for RNAi-encoding pLKO vectors due to increased size. **note: unrecombined Destination vector may not be used as a "empty vector" negative control for infections since it is not "empty" (CmR and ccDB genes). LR reactions with one of several available control ORFs (eGFP, luciferase, LacZ, etc) in Entry vectors will generate preferred controls.

Reagents:

One Shot® ccdB Survival™ 2 T1R Competent Cells
Invitrogen Cat. No. A10460

Gateway® LR Clonase® II enzyme mix
Invitrogen Cat. No. 11791-020

pLX-ORF-fwd primer:
5'-caccaaaatcaacgggactt-3'

pLX-ORF-rev primer
5'-caacaccacggaattgtcag-3'