

## Method of cloning shRNA using selection marker other than puromycin

If two genes or transcripts are to be knockdowned at the same time in one cell, pLKO\_AS2 series plasmids provides several selection makers including hygromycin, zeocin, neomycin, and EGFP (for cell sorting) that allows one to insert shRNA sequences with selection markers other than puromycin. Following protocol is one way to modify these vectors to express shRNA with alternative selection marker.

1. Choose one of the pLKO\_AS2 series plasmids (pLKO\_AS2.EGFP, pLKO\_AS2.hyg, pLKO\_AS2.neo, pLKO\_AS2.zeo) provided by the RNAi Core (please visit Vector Information in the section of Documents/ 檔案下載區 in RNAi Core website).
2. Remove IRES of EMCV by digesting with *PmeI* and *MscI*, then self-ligate and transform in *E. coli* to get IRES-deleted plasmid.
3. Digest resultant plasmid with *BamHI* and *MluI* (both sites are just upstream of CMV promoter), and dephosphorylated 5'end phospho group by alkaline phosphatase (AP), if necessary (AP reaction could be performed simultaneously along with RE digestion).
4. Purify RE- and AP-treated vector by 0.8% agarose gel.
5. Design primers to amplify shRNA expression cassette (U6 RNA polI promoter) from pLKO\_AS1.  
Primer #1: 5'-cgggatccgatcacgagactagcctc-3'  
*BamHI*  
Primer #2: 5'-ttactaaccggtacgcgtag-3'  
*MluI*
6. Digest PCR products with *BamHI* and *MluI*, and purify RE-digested products with 1% agarose gel.
7. Ligation and transformation.
8. Follow above-mentioned protocols to insert shRNA of interest into resultant plasmid.