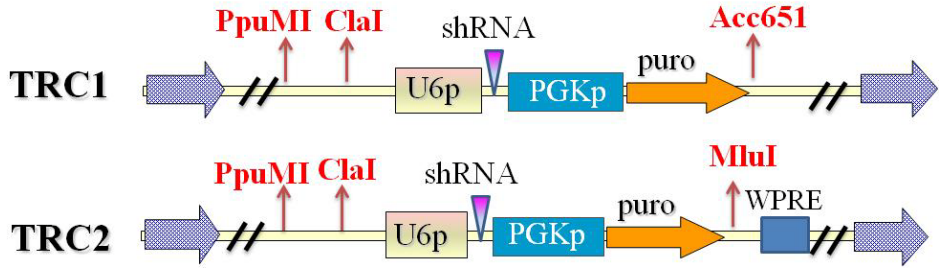
# Manual for expressing two shRNAs using one vector

## Background Information

The objective of this manual is to provide a method to construct two shRNAs in one lentivirus transfer vector that would be able to express two shRNAs upon transduced it into one cell. Although most shRNAs provided by RNAi Core have decent knockdown efficiency, there are some genes that had less satisfying knockdown efficiency in all its shRNAs available in RNAi Core. In addition, one may want to knock down multiple genes at the same time in some studies. Therefore, if one vector could express multiple shRNAs, it would be a wonderful tool in RNAi research.

The shRNA libraries of RNAi Core were cloned into pLKO.1-based TRC1 or TRC2 vectors. The maps are depicted in figures below. As shown, TRC1 and TRC2 vectors have some unique sites for inserting additional U6p shRNA expression cassettes, i.e. PpuMI, ClaI and Acc65I in TRC1 vector or PpuMI, ClaI and MluI in TRC2 vector, respectively.




To test whether one lentiviral transfer vector can express two shRNAs, we chose 4 TRC1 shRNAs (shAXL575, shAXL1040, shAXL572 and shAXL574) targeting AXL. As shown in figure 1 below, shAXL575 and shAXL1040 had excellent knockdown efficiency, whereas shRNA572 and shAXL574 had only approximately 50% KD efficiency compared to naïve A549 or control shRNA shLuc976. To combine those two lesser efficient clones into one vector, we used shAXL572 as a backbone and cloned a PCR-amplified shAXL574 fragments into Acc65I site on the vector (Figure 2). As shown in figure 1 right panel, this contruct indeed gained more knockdown efficiency than their individual clone. This data suggest that two less efficient shRNAs could increase knockdown efficiency by combining them into one vector .

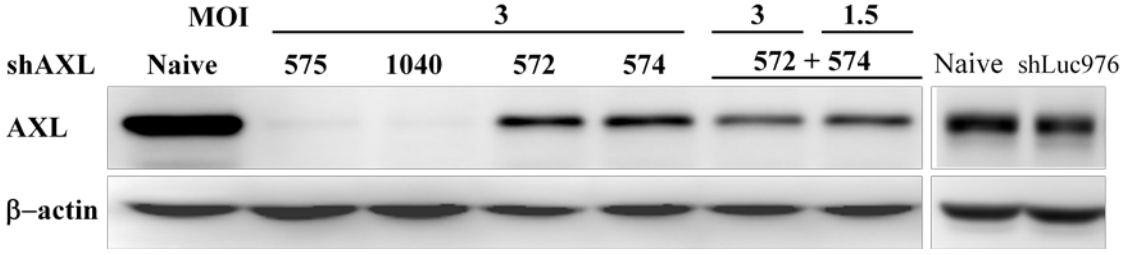



**Figure 1**


**Figure 2**

Next, we tried to build 2 shRNAs side by side (shLuc221 and shTP53 in figure 4) or 3 shRNAs on the same vector. We chose three good knockdown cfficiency clones (figure 4) for the following experiments. As shown in figure 3, individual shRNA gave rise to good KD efficiency as expected. However, when two shRNAs put together the added one (shLuc221) gave rise to less efficiency compared to shLuc221 alone; when three clones were put together shLuc221 gained lesser KD efficiency. This data suggest that (i) when additional shRNA cassette is added to the downstream of PAC ORF both shRNAs maintain an acceptable KD efficiency; (ii) when additional shRNA cassette is added upstream to the original shRNA cassette the added one gave rise to less KD efficiency.

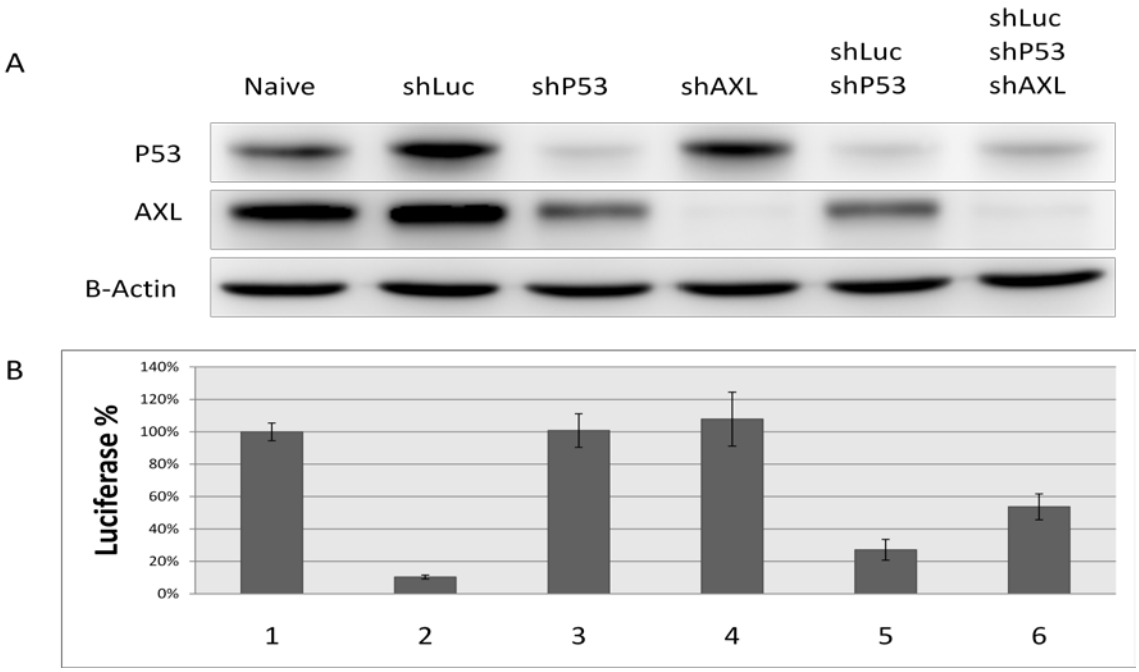



**Figure 3**


**Figure 4**

Taken these data together, we suggest that one can add additional shRNA cassette into the downstream of selection marker, i.e. Acc65I site in TRC1 vector or MluI site in TRC2 vector. Based

on this suggestion, a protocol is provided for your information.

**Experimental procedure:**

**A. Preparation of cloning vector:**

Both TRC1 and TRC2 vectors provide an additional cutting site for inserting shRNA expression cassette into the downstream of selection expression cassette, making it feasible to clone a lentivirus shRNA vector expressing two shRNAs.

1. Add 3-ug of U6p-shRNA plasmid with 10-20 units of Acc651 (for TRC1) or MluI (for TRC2) and 2 units of Alkaline Phospatase for a total volume of 100 ul
2. Incubate at 37°C for O/N.
3. Separate digested vector by agarose gel and purify cut gel (DNA) using commercial spin column.

**B. Preparation of insert:**

- PCR amplification of desired shRNA expression cassette from TRC1 or TRC2 vector by using the primer pair as follow:
  1. Acc65-Mlu-U6p/F 5'-ccaacggtaccacgcgtgagggcctatttcccatgattccttc-3'
  2. Acc65-Mlu-U6p/R 5'-ccaacggtaccacgcgtgtggatgaatactgccatttgtctc-3'

  Red : Acc651 cutting site

  Green: MluI cutting site

- Set up PCR reaction mixture

| | |
|---|---|
| 5x PCR buffer | 5 µl |
| 10 mM dNTPs | 1 µl |
| Primer F (100 µM) | 0.5 µl |
| Primer R (100 µM) | 0.5 µl |
| 10-20 ng/µl DNA template | 1 µl |
| EHF PCR polymerase (Roche) | 1 µl |
| ddH2O to | 50 µl |

**PCR parameter:**

| | |
|---|---|
| 98°C 2' | 1x |
| 98°C 15'' | |

50°C 30”
72°C 30’
25 cycles
72°C 5’ 1x
Hold at 4°C

- Check the quality and quantity of the PCR products by taking 2 µl reaction mixtures using a 1.5-2% agarose gel (product size is 376 bp).
- Clean up the PCR mix using PCR cleaning kit, then digests with10 units of ScaI[#] and 20-30 units of Acc65I (for TRC1) or MluI (for TRC2) in a reaction volume of 100 ul.
  [#]ScaI digestion would get ride of the background colonies caused by plasmid template.
- Clean up again and the PCR products are ready for ligation.
  (Option) If you still have background problem, further purify the restricted PCR products through agarose gel.

**C. Ligation**

1. Mix the following :
   - RE-cut TRC shRNA vector 50 ng
   - RE-cut PCR product 50 ng
     Adjust the volume of vector and insert with ddH2O to 5ul
   - Takara ligation mix (#6023) 5ul
2. Incubate the ligation mix at RT for 30’
3. Take 5-10 ul ligation mix for transformation (Stbl3 strain is recommended for lentiviral transfer vector transformation).
   - Note: *Total ligation mix should not exceed 10% of the competent cell volume.*

Appendex:

- Sequencing primer: 5’-gaccgcgcacctggtgcatg-3’ (locate at 3’-end PAC gene)