miRE shRNA PCR CLONING

This protocol details the PCR-based cloning of miRE shRNA cassettes into recipient miRE-compatible vectors. The cloning can be multiplexed for tandem shRNAs by using appropriate forward and reverse primers for each successive sgRNA cassette – see below.

1. Digest miRE backbone (EcoRI/XhoI), dephosphorylate the ends (CIP) and clean by column purification
2. Amplify shRNA cassette from target vector (or 97mer oligo @ 2ng/ul) using the following PCR conditions:

PCR reaction

10ul	5x buffer
5ul	dNTPs (2.5mM each)
1ul	miRE_F primer
1ul	miRE_R primer
0.5ul	Herculase II polymerase
1ul	vector template (2ng/ul)
31.5ul	Water

PCR conditions

2m	95C
25s	95C
25s	58C
30s	72C
repeat steps 2-4 x30	
5m	72C

NOTE: We routinely use Invitrogen standard synthesis oligos for this cloning as they are significantly cheaper than IDT (or other); however, we have noted that we require higher amounts of template than initially published (0.02ng; Dow et al, Nat Prot, 2012). We now use 2-5ng of oligo template for each PCR reaction.

3. Check amplification on an agarose gel. Expect a single 141bp product. Clean by column purification
4. Digest product with appropriate enzymes (2-3 hrs) and column clean. It is not usually necessary to gel extract the DNA - we have not seen significant carryover of vector template DNA into the ligation, however if you obtain high colony numbers from insert only transformation, repeat after gel extracting the shRNA insert.
5. Ligate vector and inserts at a molar ratio of ~1:5. Don't forget the vector only and insert only controls!
6. Transform competent STBL3/Stellar bacteria and plate on Amp/Carb plates. STBL3/Stellar bugs are strongly recommended for lentiviral cloning and propagation.
7. Pick colonies and screen by sequencing. *NOTE: tandem cassettes can be screened first by restriction digest*
8. Sequence clones using miRseq primer: TGTTTGAATGAGGCTTCAGTAC

9. *TANDEM*: To clone a second miRE shRNA into an existing miRE vector, digest the recipient vector with EcoRI only and amplify the new shRNA using miRE-TX_F and miRE_R primers (see below). Digest amplified shRNA with BbsI/EcoRI and ligate/screen clones as described above.

Amplification Primers

For standard miRE cloning into an empty recipient vector

miRE_F:	TGAACTCGAGAAGGTATATTGCTGTTGACAGTGAGCG **(amplifying from oligo)**
miRE_F:	AGAAGGCTCGAGAAGGTATATTGC **(amplifying from plasmid)**
miRE_R:	GCTCGAATTCTAGCCCCTTGAAGTCCGAGG

For adding additional shRNAs into the EcoRI site of an existing miRE vector

miRE-TX_F:	AGGCGCGAAGACTCAATTGAAGGCTAAAGAAGGTATATTGCTG
miRE_R:	GCTCGAATTCTAGCCCCTTGAAGTCCGAGG