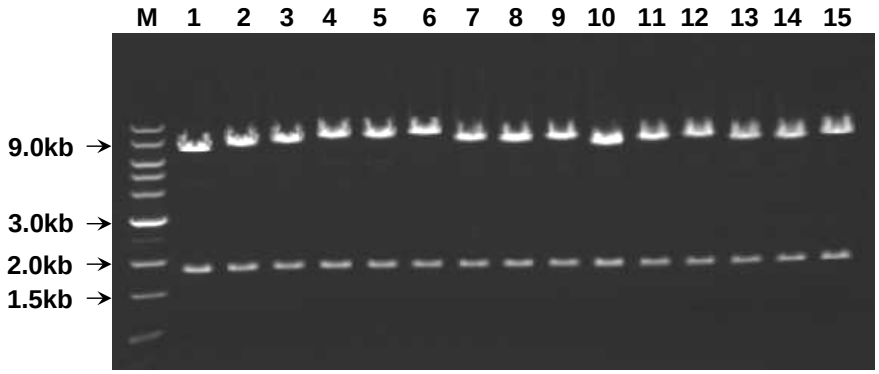
Protocol for shRNA construction-I: PCR method

Preparation of cloning vector:

1. Incubate 3 μg of shRNA cloning vector with 5 units (NEB) of *Eco*RI and 10 units of *Age*I, (double digestion) in a reaction volume of 100 μl at 37^0C for overnight (using NEB #4 buffer).
2. Take 5 μl of reaction mixture to check the digested DNA in a regular agarose gel electrophoresis. An example of such restriction analysis is shown in below:



1. LKO_TRC001	6. LKO_TRC010	11. LKO_TRC017
2. LKO_TRC005	7. LKO_TRC011	12. LKO_TRC018
3. LKO_TRC007	8. LKO_TRC013	13. LKO_TRC019
4. LKO_TRC008	9. LKO_TRC014	14. LKO_TRC020
5. LKO_TRC009	10. LKO_TRC016	15. LKO_TRC024

Figure legend: 400ng of various shRNA cloning vectors were digested with 2.5 units of *Eco*RI, 5 units of *Age*I in a reaction volume of 30 μl at 37^0C for 6 hrs (using NEB #4 buffer), and then 15 μl of mixtures mixed individually with loading buffer were subjected to electrophoresis (0.5% agarose gel containing 0.1μg/ml of EtBr, run at 50 volts in 0.5X TBE running buffer containing 0.1μg/ml of EtBr) for 2 hours. As shown in this figure, the 1.9 kb stuffer sequence was completely digested out under this reaction condition, an indication of complete digestion of those vectors.

Please note that *Eco*RI is easy to trigger star activity, an altered or relaxed specificity of the enzyme (please refer to NEB catalog and technical reference for how to avoid star activity of restriction enzymes).

3. Resolve restriction enzymes digested shRNA cloning vector by 0.7% agarose gel (mix of 0.35% regular agarose plus 0.35% low melting agarose recommended).
4. Purify DNA from gel according to the instructions of Roche or Qiagen DNA elution kit and elute it with 50 μl of autoclaved 0.1X TE buffer.

Preparation of insert (PCR product):

1. Design and order a long oligonucleotide as a PCR template for amplifying shRNA sequence you desired to clone (see appendix 1 for how to design shRNA oligonucleotide sequence).
2. Set up PCR reaction:

shRNA oligonucletide (50μM)	1μl
Forward primer (100μM)	2μl
Reverse primer (100μM)	2μl
KAPAHiFi DNA Polymerase	1μl
5X GC buffer	10μl
2mM dNTPs	5μl
d.water final volume to	50μl

3. PCR parameter:

Step1→ 95^{0}C, 1cycle	5-min
Step2→ 95^{0}C	15 Sec
Step3→ 65^{0}C	30 Sec
Step4→ 72^{0}C	20 Sec
(Repeat step 2 to step 4 for another 2 cycles)	
Step5→ 72^{0}C, 1 cycle	5-min
Hold at 20^{0}C	

4. Purify PCR product using MinElute Gel Extraction Kit (Qiagen), and elute it with 80 μl of autoclaved 0.1X TE buffer.
5. Digest eluted PCR product with *Bsm*BI and incubate at 55^{0}C in the incubator rather than in water bath for O/N (see appendix 2 for the restriction pattern of *Bsm*BI restriction enzyme).

   Digestion condition:

Eluted PCR product	80μl
NEB #3 10X buffer	10 μl
*Bsm*BI (10U/μl; NEB)	2 μl
d.water final volume to	100μl

6. Purify *Bsm*BI-digested PCR product using MinElute Gel Extraction Kit (Qiagen) and elute it with 20 μl of 0.1X TE buffer. (Recovery rate for 55-60 bp DNA is satisfactory if using this kit.).
7. Set up the ligation reaction (use of 2 μl vector and 4 μl of PCR products, respectively).
8. Transform in E. coli **(Stbl III [Invitrogen] recommended; Lentivector is hard to transform into DH5α)**.

Protocol for shRNA construction-II: annealing method

1. Design sense (tail with CCGG [*Age*I cohesive end] sequence at the 5' end) and antisense (tail with AATT [*Eco*RI cohesive end] sequence at the 5' end) shRNA oligonucleotide as follows:

   If consider **cgcatacgacgattctgtgat** as the target sequence, then sense (up-strand sequence of following example) and antisense (low-strand sequence of following example) oligonucleotide, respectively, will be as follows:

   target sequence/passenger strand siRNA sequence/guide strand

   ccggcgcatacgacgattctgtgat**ctcgag**atcacagaatcgtcgtatgcgttttt

   gcgtatgctgctaagacacta**gagctc**tagtgtcttagcagcatacgcaaaaa**ttaa**

   ctcgag: loop sequence of shRNA

   Please note that the design is in concert with TRC shRNA library's design.

2. Order oligonucleotides with 200 nmole scale and OPC or PAGE purification (oligonucleotide produced by Mission Biotech recommended).
3. Dissolve oligonucleotides into 100 μM with autoclaved distillated water.
4. Prepare 10X annealing buffer:

   1M K-acetate

   0.3M HEPES-KOH pH7.4

   20 mM Mg-acetate
5. Set up annealing mixture:

Sense oligo	9 μl
Antisense oligo	9 μl
10X annealing buffer	2 μl

6. Anneal mixture by PCR machine using the following parameters:

   95^0C, 78^0C, 74^0C, 70^0C, 67^0C, 63^0C, 60^0C, 56^0C, 53^0C,

   50^0C, 48^0C, 46^0C, 44^0C, 42^0C, 40^0C, 39^0C,37^0C,

   36^0C, 35^0C, 34^0C, 33^0C, 32^0C, 31^0C----------5 min in each step

   30^0C, 28^0C, 26^0C, 24^0C, 22^0C, 20^0C----------10 min in each step

   Hold at 4^0C
7. Dilute annealing mixture 50x or 100x with ddH_2O
8. Set up ligation reaction mixture and ligation for O/N:

RE-restricted shRNA cloning vector (Prepare vector followed aforementioned protocol)	2 μl
Annealed oligonucleotides	2 μl
10X ligation buffer	1 μl
ligase (1 unit/ μl)	1 μl
d.water final volume to	10 μl

9. Take 5-µl ligation mixture and transform it into E. coli competent cells (Stbl III).

Appendix 1: Design of shRNA oligonucleotide sequence for PCR amplification:

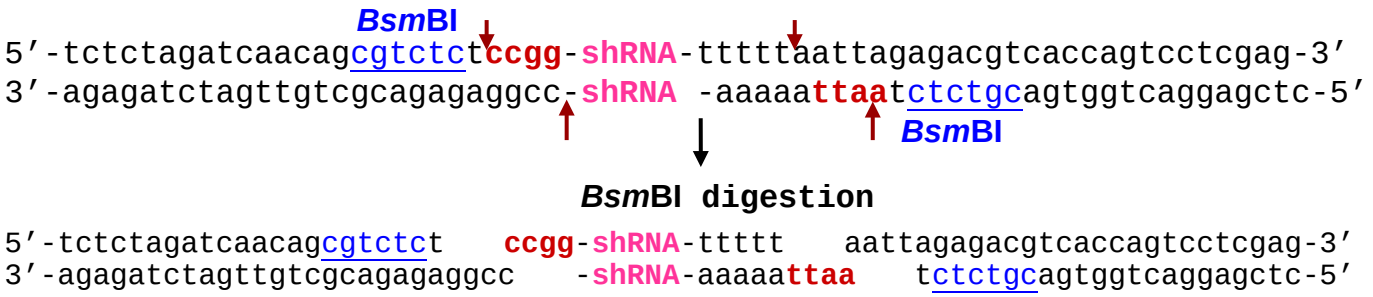
As shown in following example, shRNA sequence plus TTTTT is flanked with common 5' and 3' end sequences (as indicated by bold sequences in given example) that include two *Bsm*BI recognition sites (the inclusion of *Bsm*BI in the design is described in Appendix 2). The design of shRNA sequence is described in protocol II: annealing method for shRNA construction.

5'-**tctctagatcaacagcgtctctccgg**-**shRNA**-ttttt**aattagagacgtcaccagtcctcgag**-3'

- Primers for PCR amplification of shRNA containing sequence:
  shRNA-all/F: 5'-tctctagatcaacagcgtctc-3'
  shRNA-all/R: 5'-ctcgaggactggtgacgtctc-3'

Appendix 2: Restriction pattern of *Bsm*BI restriction enzyme

*Bsm*BI-digested DNA will produce two 5' protruding ends with any sequences by your design (please note that protruding sequences in the following diagram [generated by *Bsm*BI] can be ligated to *Age*I and *Eco*RI -restricted cohesive ends, respectively):



Appendix 3: Sequencing primers

1. LKO_shRNA/F (forward primer):
   5'-acaaaatacgtgacgtag-3' (for sequencing forward strand of shRNA)
2. LKO_shRNA/R (reverse primer):
   5'-ctgttgctattatgtctac-3' (for sequencing reverse strand of shRNA)

Appendix 4: Sequencing method

1. First try regular sequencing kits in the presence of 5% DMSO to determine shRNA sequence.
2. The failure of method 1 could be due to secondary structure of shRNA. If so, then try dGTP BigDye V1.1 kit or equivalent product to determine shRNA sequence.

Appendix 5: Terrific Broth 配方 (Please refer to Molecular cloning for detailed)

Tryptone	12 g/L
Yeast extract	24 g/L
Glycerol(100%)	4 ml/L
KH_2PO_4	2.31 g/L
K_2HPO_4	12.54 g/L

Ampicillin or Carbenicillin final concentration：100mg / L