Protocol for construction of miRNA sponge

Introduction

A class of small RNA molecules termed microRNAs (miRNAs) play a pivotal role in development as well as in balancing normal physiology in multicellular organisms (Ambros, 2004; He and Hannon, 2004). The expression of miRNA is frequently deregulated during tumorigenesis, and some miRNAs are involved in cell proliferation and survival (Dalmay and Edwards, 2006). A body of evidence indicates that the altered expression of miRNAs in cancer may involve either loss or amplification of genomic sequences (Dalmay and Edwards, 2006). Thus, miRNAs may function as tumor suppressors or oncogenes. A recent genetic screen using miRNA library demonstrated that miRNA-372 and miRNA-373 function as oncogenes possibly through direct inhibition of the expression of tumor suppressor LATS2 (Voorhoeve et al., 2006). In a word, the study of microRNA is extremely important in understanding the network of gene regulation as well as cancer biology. In view of this, we are interested in developing tool(s) to compete with the effective microRNA molecules in cells; as a result, the amounts of microRNA would be reduced which may shed light into the understanding of the function of microRNAs in cells.

There are two major approaches to block microRNAs' function, namely microRNA sponge (Ebert et al., 2007; Brown and Naldini, 2009; Scherr et al., 2007; Hammond, 2007) and anti-microRNA oligonucleotide (Boutla et al., 2003; Hutzvanger et al., 2004; Krutzfeldt et al., 2005; Weiler et al., 2006; Esau, 2008). We are particularly interested in the approach of microRNA sponge. The effective molecules produced by microRNA sponge method could be continuously synthesized in the cells, thus can give rise to long-term inhibition of the microRNA function. The method of microRNA sponge was established by Sharp's laboratory in 2007 (Ebert et al., 2007). Afterwards, several modifications have been reported (Ebert and Sharp, 2010; F.C. Tay et al., 2015). We take advantage of these reports to fine tune the method of microRNA. The protocol is described as follows.

Build up a lentivirus-based microRNA sponge vector

A lentiviral vector, named AS7w.mCherry-CMV.d2EGFP, expressing miRNA sponge was constructed. The miRNA sponge sequences were inserted into the 3'UTR of destabilized GFP reporter gene driven by the CMV immediate early promoter. The mCherry gene driven by hPGK promoter in the construction was used as a control for tracking transfection or infection efficiency. Cells can be enriched by sorting d2EGFP/mCherry positive signal or be selected by puromycin for establishing stable cell. This vector contains 1.9 kb stuffer inserted in between *Eco*RI and

*Xma*I cloning sites for inserting multiple miRNA binding sites (microRNA sponge sequences). This vector can be used for inhibiting miRNA function by transient transfection or by transducing with packaged lentivirus.

Overview of microRNA sponge construction

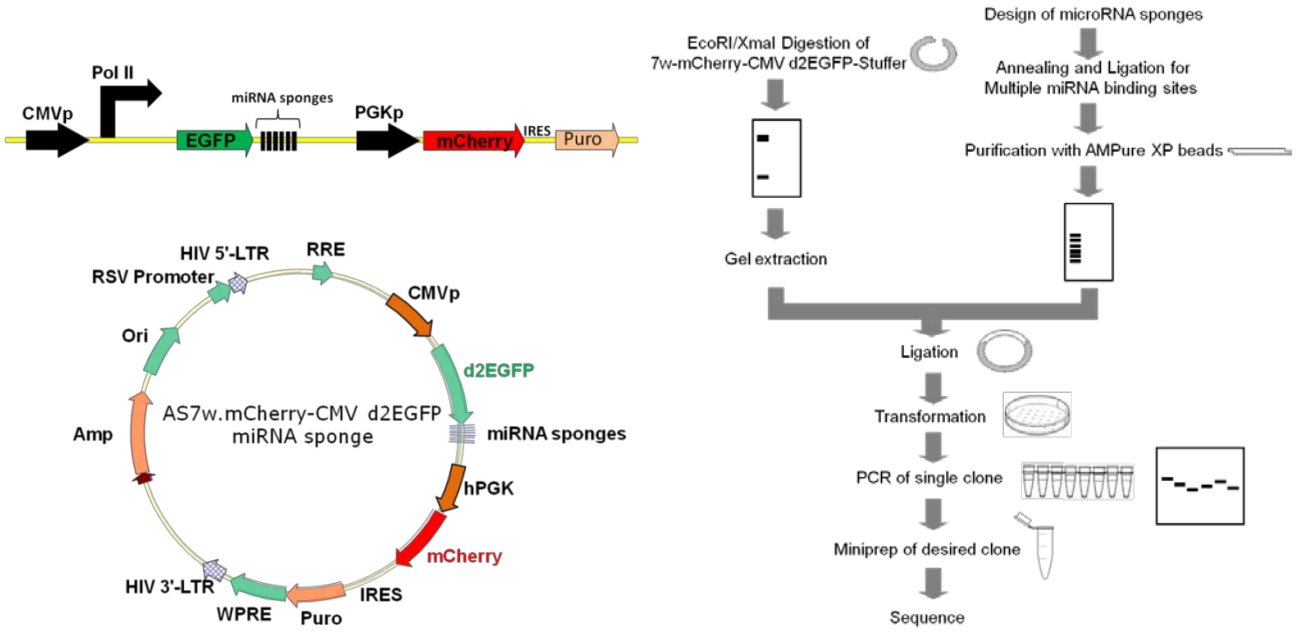


Fig. 1: (Left): Maps of the AS7w.mCherry-CMV.d2EGFP miRNA sponge lentiviral vector. (Right): Workflow of inserting multiple copies of miRNA sponge sequence into the miRNA sponge cassette as indicated at the left map.

Protocol for miRNA sponge construction

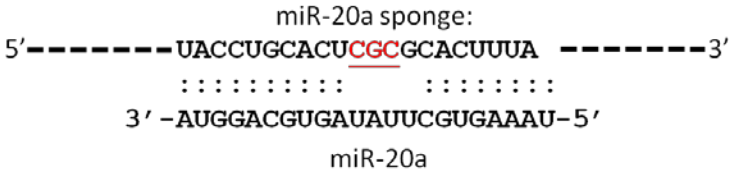
I. Preparation of cloning vector

1. Digest 3 µg of miRNA sponge cloning vector with 5 units (NEB) *Eco*RI and 10 units of *Xma*I, (double digestion) in a reaction volume of 100 µl at 37°C for overnight (using NEB CutSmart™ Buffer).
2. Take 5 µl of reaction mixture to check the digested DNA in a regular agarose gel electrophoresis. The 1.9 kb stuffer sequence will be completely digested under this reaction condition, an indication of complete digestion of the vector. 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. Separate digested vector by 0.7% agarose gel (mix of 0.35% regular agarose plus 0.35% low melting agarose is recommended).
4. Purify DNA from gel according to the instructions of Roche or Qiagen DNA elution kit and elute it

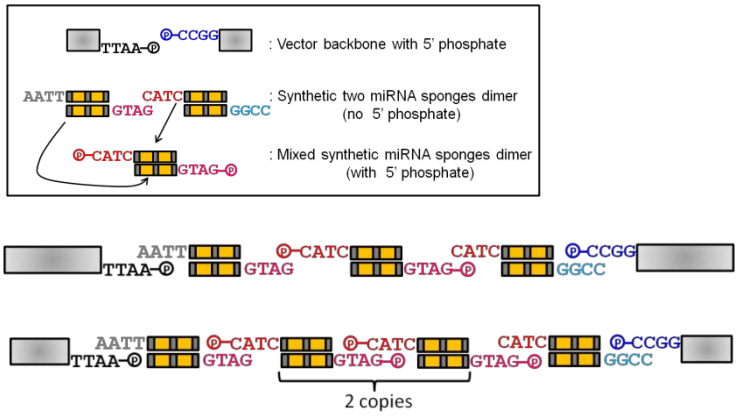
with 50 µl of autoclaved 0.1X TE buffer.

II. Preparation of Insert

As shown in above vector map, the microRNA sponge cassette is designed located at the downstream of EGFP reporter gene. To avoid microRNA sponge containing reporter mRNA being degraded by microRNA, the sponge sequence would be designed to contain an MBS (middle bulge sequence) with a 3-nucleotide (nt) bulge located at the central region. For example, if the target is miR-20a (5'-UAAAGUGCUUAUAGUGCAGGUA-3') the sponge sequence would be designed to be 3'-TACCTGCACTCGCGCACTTTA-5' (underline sequences are bulge sequence). The formed heteroduplex is depicted as below (red sequences are bulge sequence):



Figures below show the schematic representations how to prepare multiple copies of microRNA sponge for ligation.



Based on the tailed protruding ends, design 3 pairs of complementary miRNA sponge oligonucleotide sequences with different protruding ends as depicted below:

For F1/R1

```
5'-AATTNNNNNNNNNNNNNNNNNNNN-3'
     3'-NNNNNNNNNNNNNNNNNNNNGTAG-5'
```

F1: tail with **AATT** [would ligate to *Eco*RI cohesive end on vector] sequence at the 5'-end
R1: tail with **GATG** sequence at the 5'-end

For F2/R2

```
5′-CTACNNNNNNNNNNNNNNNNNNN-3′
      3′-NNNNNNNNNNNNNNNNNNNGGCC-5′
```

F2: tail with **CTAC** sequence at the 5'-end
R2: tail with **CCGG** [would ligate to *Xma*I cohesive end on vector] sequence at the 5'-end

Thus, the F1/R1 and F2/R2 pairs of oligonucleotide would be designed as follows:

F1/R1
Sense strand oligo (F1): 5'-**AATT**TACCTGCACTCGCGCACTTTA-3'
Antisense strand oligo (R1): 5'-**GATG**TAAAGTGCGCGAGTGCAGGTA-3'

F2/R2
Sense strand oligo (F2): 5'-**CATC**TACCTGCACTCGCGCACTTTA-3'
Antisense strand oligo (R2): 5'-**CCGG**TAAAGTGCGCGAGTGCAGGTA-3'

Detailed procedures are described as follows:

1. Order 200 nmole scale oligonucleotides that shall be purified by OPC or PAGE (Mission Biotech's products are recommended).

2. Dissolve oligonucleotides into 100 μM with autoclaved distilled water.

3. Prepare 10X annealing buffer:

1 M NaCl
100 mM Tris-HCl pH 8.0
10 mM EDTA pH 8.0

4. Set up annealing mixture (making sponge duplex of F1/R1, F2/R1, F2/R2):

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

5. Anneal mixture by PCR machine using the following parameters:
   95℃, 78℃, 74℃, 70℃, 67℃, 63℃, 60℃, 56℃, 53℃,
   50℃, 48℃, 46℃, 44℃, 42℃, 40℃, 39℃,37℃, 36℃,
   35℃, 34℃, 33℃, 32℃, 31℃----------5-min in each step
   30℃, 28℃, 26℃, 24℃, 22℃, 20℃----------10-min in each step
   Hold at 4℃

6. Add phosphate group to the 5'-end of F2/R1sponge duplex using Polynucleotide Kinase (PNK).

   Reaction mixture set up:

Annealed F2/R1 duplex	2 µl
T4 PNK	1 µl
10X T4 DNA ligase buffer	2 µl
ddH2O	15 µl
	20 µl

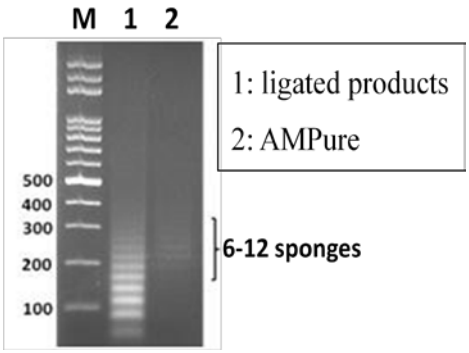
   Using PCR machine to perform kinasing reaction by using the following parameters:
   37℃----------30-min
   80℃, 75℃, 70℃, 65℃, 60℃, 55℃, 50℃----------20-min in each step
   Hold at 4℃
   * PNK reaction buffer (NEB) DOES NOT contain the ATP. It requires for the phosphorylation reaction to occur. We use T4 ligase buffer as an ATP source.

7. Set up ligation reaction mixture as follows:

   (i) Use ratio of F1/R1: F2/R1: F2/R2 = 1:2:1 or 1:4:1

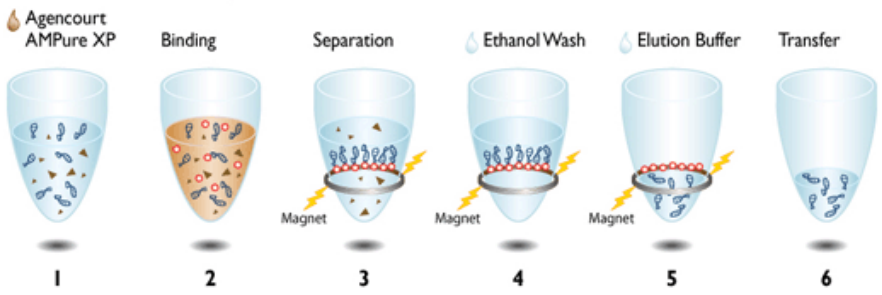
PNK-duplex oligonucleotide (F2/R1) mixture----------	20 µl
duplex oligonucleotide (F1/R1)----------	1 µl
duplex oligonucleotide (F2/R2)----------	1 µl
T4 DNA ligase ----------	1 µl
10X T4 DNA ligase buffer----------	1 µl
ddH2O to ----------	6 µl
Total	30 µl

   (ii) Ligate the mixture at 14^{o}C for O/N.
   (iii) A typical ligation pattern is showed in figure below:



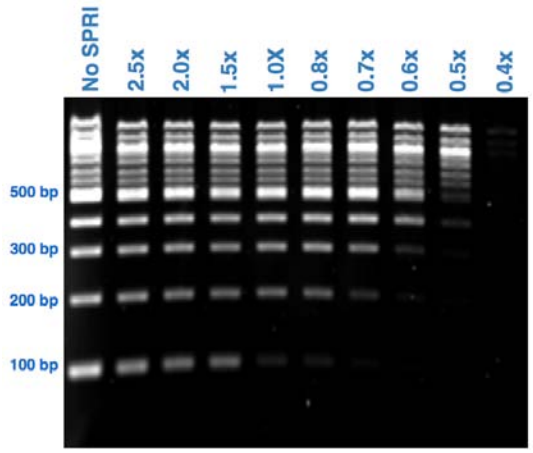
8. Purify ligated products by using AMPure XP kit (Beckman coulter Cat. A63880/A63881/A63882).

(i) The procedures are followed manufacture's manual and briefly depicted and described as follows (The ratio of ligated products/AMpure XP beads is 1:0.8):



1. Add 70 µl 1X TE buffer with 30 µl ligated products to make a total volume of 100 µl.
2. Add 80 µl AMpure XP beads into the mixture (If the ratio of ligated products/AMpure is 1:0.8); pipetting up and down 10 times to mix well; then, incubate at RT for 15 min.
3. Separate bound DNA by placing the mixture on the magnetic stand at RT for 5 min.
4. Discard the supernatant (leave the tube on the magnetic stand while performing 80 % EtOH wash step), then wash with freshly prepared 80 % EtOH without disturbing the beads, incubate at RT for 30 seconds and discard the supernatant again. Repeat this step once.
5. Dry the tube by standing it at RT for 15 min. Resuspend the dried pellet with 30 µl 0.1X TE, incubate at RT for 2 min.
6. Place the tube on the magnetic stand at RT for 5 min, and then transfer the clear supernatant to a new PCR tube. The products are ready for ligation.

(ii) Figure below shows the biding profile using different ratios of SPRI beads (Beckman Coulter) as indicated.



III. Ligation and determine the inset size of miRNA sponge

1. Set up ligation mixture
   Digested vector---------- 50-100 ng
   Insert---------- l μl
   T4 DNA ligase (5U/ul)---------- 1 μl
   ddWater to---------- 10 μl

2. Incubate at RT for 3 hr or O/N.

3. Take 5 μl ligation mixture into E. coli competent cells (Stbl III).
   (Stbl III [Invitrogen] is recommended)

4. Use PCR method to determine insert size, usually pick 5~10 colonies for colony PCR.
   PCR parameters and primers are listed below.

miRNA sponge primers	Sequence (5'→3')
Forward	AGGTGGAGGAGCAGGATGAT
Reverse	CTATTCTTTCCCCTGCACTG

5. Expected size and gel pattern are showed below.

Copy No. (n)	size (26n+4)	Size of PCR product
1	30	247
2	56	273
3	82	299
4	108	325
5	134	351
6	160	377
7	186	403
8	212	429
9	238	455
10	264	481
11	290	507
12	316	533
13	342	559
14	368	585
15	394	611
16	420	637

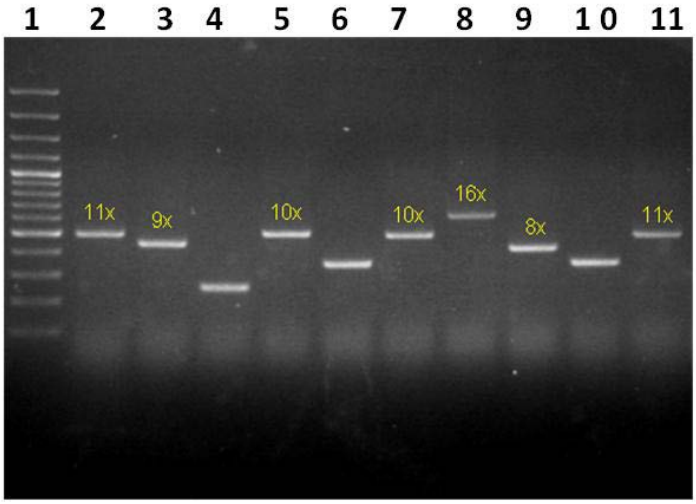


Figure legend: Left Table: Expected size of various copies of sponge;
Right panel: Lane 1: 100bp Marker, Lanes 2 to 11: PCR products with different copies of miRNA sponge.

6. Clones with desired copies of sponge subject to mini-prep and sequencing to determine sponge sequence.

References:

1. Ambros, V. (2004). The functions of animal microRNAs. Nature 431, 350-355.
2. He, L., and Hannon, G. J. (2004). MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 5, 522-531.
3. Dalmay, T., and Edwards, D. R. (2006). MicroRNAs and the hallmarks of cancer. Oncogene 25, 6170-6175.
4. Voorhoeve, P. M., le Sage, C., Schrier, M., Gillis, A. J., Stoop, H., Nagel, R., Liu, Y. P., van Duijse, J., Drost, J., Griekspoor, A., et al. (2006). A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. Cell 124, 1169-118
5. Ebert MS, Neilson JR, Sharp PA (2007). MicroRNA sponges: Competitive inhibitors of small RNAs in mammalian cells. Nat Methods 4: 721–726.
6. Brown B.D. and L. Naldini (2009). Exploiting and antagonizing microRNA regulation for therapeutic and experimental applications. Nat Rev Genet 10, 578-585.
7. Scherr M, Venturini L, Battmer K, Schaller-Schoenitz M, Schaefer D, Dallmann I, Ganser A, Eder M (2007). Lentivirus-mediated antagomir expression for specific inhibition of miRNA function. Nucleic Acids Res 35(22):e149

8. Scott M Hammond (2007). Soaking up small RNAs. Nat Methods 4: 694–695.
9. MARGARET S. EBERT and PHILLIP A. SHARP (2010). MicroRNA sponges: Progress and possibilities. RNA 16(11), 2043-50.
10. Felix Chang Tay, Jia Kai Lim, Haibao Zhu, Lau Cia Hin, Shu Wang (2015). Using artificial microRNA sponges to achieve microRNA loss-of-function in cancer cells. Adv Drug Deliv Rev 81: 117-27.
11. Boutla A, Delidakis C, Tabler M (2003). Developmental defects by antisense-mediated inactivation of micro-RNAs 2 and 13 in Drosophila and the identification of putative target genes. Nucleic Acids Res 31(17): 4973-80.
12. Hutvágner G, Simard MJ, Mello CC, Zamore PD (2004). Sequence-specific inhibition of small RNA function. PLoS Biol 2(4):E98. Epub 2004 Feb 24.
13. Krützfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, Stoffel M (2005). Silencing of microRNAs in vivo with 'antagomirs'. Nature 438(7068): 685-9. Epub 2005 Oct 30.
14. Weiler J, Hunziker J, Hall J (2006). Anti-miRNA oligonucleotides (AMOs): ammunition to target miRNAs implicated in human disease? Gene Ther 13(6): 496-502.
15. Esau CC (2008). Inhibition of microRNA with antisense oligonucleotides. Methods 44(1):55-60.