How to Select Optimal siRNA Target Sites Using SOS-Based Vectors

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I. Select Desirable Vectors:

There are at least three types of SOS-based vectors in our lab:

1) **pSOS-HUS and pSOS-HUD**: the first generation SOS vectors; driven by both H1 and U6 promoters.
2) **pSEB-HUS (a.k.a., pSOS) and pSEB-HUD**: retroviral-based SOS vectors; can be used for both selection and making stable knockdown cell lines; there may be different versions of antibiotics selection markers, and/or dual U6 promoter-driven vectors. These are preferred vectors for siRNA site validation.
3) **pASV-HUS (a.k.a., pSES)**: adenoviral-based SOS vector; not for selecting and validating; only used to make adenoviruses to express the already validated effective siRNA sites; co-expressing RFP marker; fully compatible with the current AdEasy system.

II. Clone Target Gene/Sequence into SOS-Based Vectors (using pSEB-HUS or pSOS as an example):

Clone the coding region or the region containing potential siRNA sites (***see below***) of your target gene into multiple cloning sites. Although your gene/sequence will not make any fusion proteins with the eGFP (i.e., GFP has a stop codon), you should clone your gene in the same 5' to 3'-end direction with respect to the eGFP coding region. The resultant vector is designed as ***pSEB1-Gene-HUS***.

III. Design Potential siRNA Target Sites (using pSEB-HUS, or pSOS as an example):

There are numerous commercial websites that can help you to design potential siRNA sites. We have the best success with **Dharmacon**'s ***siDESIGN***: http://www.dharmacon.com/sidesign/.

Other sites include Ambion (http://www.ambion.com/techlib/resources/RNAi/index.html), IDT (http://www.idtdna.com/Scitools/Applications/RNAi/RNAi.aspx), MWG BioTech (http://www.mwg-biotech.com/html/s_synthetic_acids/s_rna.shtml), or Whitehead Institute's siRNA Selection (http://jura.wi.mit.edu/bioc/siRNAext/).

The target site can be 19 to 27 nucleotides. We have very good luck with 19 nucleotides. When design the actual oligos, please remember to add **one extra "A"** in front of the target site, and **four extra "Ts"** at the 3'-end of the target site, as follows (for HUS vectors):

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Top Strand         5'-ANNNNN......NNNNTTTT-3'      (n=19~27nt)
Bottom Strand  3'-TTTTNNNNN......NNNNA-5'
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You can make them from any oligo synthesis vendors. We order oligos from IDT.

IV. Clone Oligo Cassettes into SOS Vectors (using pSEB-HUS, or pSOS as an example):

A) Preparation of the Annealed Oligo Cassettes:

1) Dissolve each of oligo (sense and anti-sense strands) in dd-H2) to make stocks at final concentration of 1.0ug/ul.
2) Transfer 20ul each to a new screw cap 1.7ml microfuge tube, and mix well.
3) Boil the oligo mixture for 5-10 minutes.
4) Cool the mixture down at room temperature (usually taking 5-10 min).
5) Transfer **10ul** of annealed oligo mixture to a new 1.7ml microfuge tube, and add **490ul** dd-H2O (i.e., **1:50 dilution**). This is your annealed oligo cassette ready for ligation reaction.

NOTE: We have tried to use different dilutions of annealed oligo cassettes, ranging from 1:10 to 1:100 dilutions. The common mistake for oligo cassette cloning is to use too much of the oligo cassette, which will **actually reduce** the ligation efficiency due to multimerization among the substantially excessive cassettes.

B) Preparation of Vector (e.g., pSEB-HUS)**:**

1) Set up Sfi I digestion reaction as follows:

pSEB1-Gene-HUS (miniprep DNA)	5.0ul
dd-H2O	80.0ul
10X Buffer #2 (NEB)	10.0ul
100X BSA (NEB)	2.0ul
Sfi I (NEB)	3.0ul

2) Incubate at 50^{o}C for 30 min. followed by ethanol precipitation and 70% ethanol washing.
3) Dissolve the DNA in 12ul dd-H2O. Ready for ligation reaction or kept at -20C.

C) Ligation Reaction and Transformation**:**

1) Set up ligation reaction as follows:

5X Ligation Buffer (Invitrogen)	3.0ul
dd-H2O	9.0ul
pSEB1-Gene-HUS cut with *Sfi* I	1.0ul
Annealed Oligo Cassette (1:50)	1.0ul
T4 DNA Ligase (Invitrogen)	1.0ul

2) Incubate at 16C for 2-4hrs.

3) Perform digestion reaction (100ul reaction) of the ligation mix with *Sfi* I or *Not* I, or *BstX* I (usually 2ul enzyme at 37C for 15-30min).

4) Perform ethanol precipitation and electroporation using the standard protocols in our lab.

5) You can use U6 Fwd primer and the anti-sense oligo of your cassette to perform PCR screening of the recovered colonies.

6) You can also use U6 Fwd primer to sequence the cloned cassette. The resultant vector is designed as ***pSEB2-siGENE***.

V. Select and Validate Optimal siRNA Sites in HEK-293 cells:

1) Perform Wizard miniprep (using equivalent commercial kits) for pSEB2-siGENE plasmid DNA. Also, purify the pSEB1-GENE-HUS vector, which will be used as the 100% GFP signal control.

2) Plate HEK-293 cells in 12-well plates at ~2-4 hours prior to transfection.

3) Transfect the purified pSEB2-siGENE or pSEB1-GENE-HUS DNA into subconfluent 293 cells, using LipofectAIMINE. NOTE: Other cell lines can also be used.

4) Check GFP signal intensity at days 2 to 7 after transfection. The GFP signal intensity of the pSEB1-Gene-HUS transfection will serve as a non-knockdown negative control. The GFP signal can also be measured quantitatively using the Modulus Fluorometer. **NOTE**: We usually see substantial silencing effect at 3-5 days post transfection.

5) For further validation of the silencing effect, one can perform Western blotting of the silenced target gene or do functional analysis of the target gene.

VI. Convert SOS Vectors into siRNA Expression Vectors:

Once one or a few siRNA target sites are validated, the target gene sequence can be removed from the pSEB2-siGENE vector via *Pac* I digestion/re-ligation, resulting in ***pSEB3-siGENE*** vector. This will converted pSEB2-siGENE into a siRNA expression vector, and the GFP, in this case will serve as a marker for monitoring the transfection efficiency. The pSEB3-siGENE vector can also be used to generate retrovirus and to make stable cell lines.

For making **adenoviral vectors**, the oligo cassettes of the validated target sites will be subcloned into the *Sfi* I site of **pASV-HUS (a.k.a., pSES)**, followed by DNA sequencing verification. Recombinant adenovirus expressing siGENE can then be produced by following the standard AdEasy protocol.