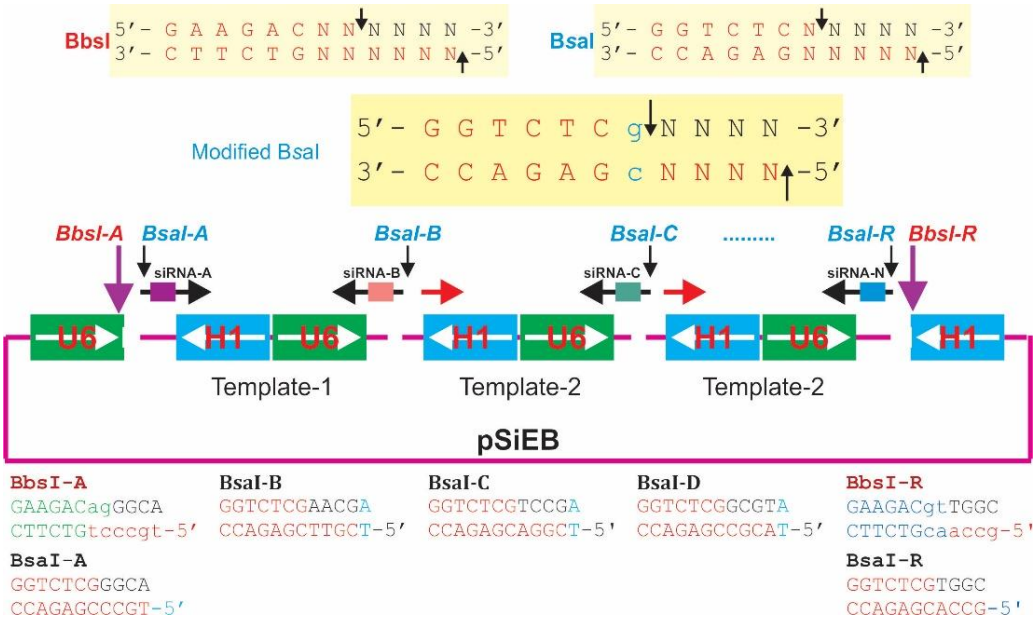
How to Use the FAMSi System to Construct Multiplex siRNA Vectors

(Fang He @ 03/28/2020; Commented by TCH)

[*He F et al. Molecular Therapy-Nucleic Acids 22:885-899; DOI: doi.org/10.1016/j.omtn.2020.10.007*]

GENERAL NOTE: The Fast Assembly of Multiplex siRNA (**FAMSi**) system allows one-step assembly of multiple siRNA expression cassettes driven by U6 and H1 promoters (see the diagram). This simplified system only requires the use of two restriction enzymes, Bbs*I* and Bsa*I* (although Hind*III* can be used to cut ligation products to reduce background). To accomplish this, one has to use the two specific template vectors (Temp-1 and Temp-2; see Appendix-2). Lastly, different primer designing guidelines need to be followed for constructing 3, 4, or 5 siRNA sites (see Appendix-1).



A. Preparation of the Destination Vector with BbsI Digestion

1) Set up **Bbs*I*** digestion of the destination vector pSiEB in 100ul reaction system:

10x CutSmart	10µl
pSiEB miniprep DNA	5µl
ddH_2O	82µl
Bbs1	3µl
Total	100µl

2) Incubate at 37°C for 30min.
3) Perform ethanol precipitation.
4) Dissolve the DNA pellet in 20µl ddH_2O
   [Optional: checking 2µl on agarose gel to guestimate rough concentration of digested product].
5) Keep the BbsI-digested pSiEB vector at -20°C or -80°C till use.

B. Preparation of Individual siRNA-Containing Fragments

Preparation of individual siRNA fragments by two-stage PCR amplification: The inserted siRNA fragments will be obtained by two-step PCR reaction. The 1st PCR products will be used as templates for the 2nd PCR reaction to get the final siRNA Fragments products.

B-1. 1st Round PCR

The first siRNA fragment is amplified by using **pH1U6-T1** as the template, while all other siRNA fragments are amplified by using **pH1U6-T2** as the template.
[**NOTE**: Fusion Hi-Fi PCR system is highly recommended]

1) Prepare the following Fusion Hi-Fi PCR reaction system for each siRNA Fragment:

ddH_2O	12.4μl
5x Phusion HF Buffer	4.0μl
10mM dNTPs	0.4μl
DMSO	0.6μl
Primer#1 (300ng/μl)	0.2μl
Primer#2 (300ng/μl)	0.2μl
Phusion Hi-Fi DNA Pol	0.2μl
pH1U6 template (miniprep)	2.0ul
Total	20μl

2) Add 10μl mineral oil;
3) Run two-stage PCR as follows:

95.0°C for 00:04:00
92.0°C for 00:00:30
45.0°C for 00:00:30 } -1°C/cycle, 3 cycles
72.0°C for 00:00:30

92.0°C for 00:00:30
65.0°C for 00:00:30 } 5~20 cycles
72.0°C for 00:00:30

72.0°C for 00:05:00
12.0°C forever

B-2. 2nd Round PCR

1) Add **60μl ddH_2O** to the 1st round PCR product (i.e., total vol. = 80ul);
2) Prepare 100μl **Hi-Fi PCR reaction system** as the follows:

ddH_2O	62.0μl
5x Phusion HF Buffer	20.0μl
10mM dNTPs	2.0μl
DMSO	3.0μl
Primer#1 (300ng/μl)	1.0μl
Primer#2 (300ng/μl)	1.0μl
Phusion Hi-Fi DNA Pol	1.0μl
Diluted 1st Rd PCR product	10.0ul
Total	100μl

3) Divide into **Five** PCR tubes (i.e., 20μl/tube);
4) Add 10μl mineral oil to each tube;
5) Run PCR:

95.0℃ for 00:03:00
92.0℃ for 00:00:30
58.0℃ for 00:00:30 } 5~20 cycles
72.0℃ for 00:00:30

72.0℃ for 00:05:00
12.0℃ ∞

6) Check 10µl of the 2nd Rd PCR product on agarose gel. If the PCR products for all siRNA fragments are correct, pool the five tubes of PCR products and perform ethanol precipitation, dissolve the plate in pellet in 20µl ddH_2O.

C. BsaI Digestion and Pre-Assembly of the siRNA Fragments

C-1. Bsa*I* Digestion of the siRNA Fragments

1) **Bsa*I*** digests each siRNA fragment as the following system

PCR-amplified siRNA Fragment	10.0ul
NEB 10x CutSmart Buff	10.0µl
ddH_2O	77.0µl
Bsa*I*	3.0µl
Total	100µl

2) Incubate at 37°C for 30min.
3) Perform ethanol precipitation.
4) Dissolve the DNA pellet in 10-20µl ddH_2O. Check 2µl on gel to compare and estimate relative concentrations of all PCR-amplified siRNA fragments.

C-2. Pre-Assembly of the siRNA fragments

1) The ligation reaction system as the follows (using 3-fragment assembly as an example)

5x Invitrogen Ligase buffer	3.0µl
BsaI-cut siRNA Fragment 1	1.0µl (may vary depending on concentration)
BsaI-cut siRNA Fragment 2	1.0µl (may vary depending on concentration)
BsaI-cut siRNA Fragment 3	1.0µl (may vary depending on concentration)
ddH_2O	8.0µl
T4 DNA Ligase	1.0 µl

2) Incubate reactions at room temperature for 30min.
3) At the end of the above reaction, add 3µl 6x Loading Buffer (DNA sample buffer), and load to **0.6%-0.8%** agarose gels. The gel should be resolved at **60-70V for 40-60min**.
4) Isolate the fully assembled DNA fragment from the gel using homemade Magic Column.
5) PC-8 extraction twice ⟶ ethanol precipitation/washing ⟶ dissolve the pellet in 12µl ddH_2O.

D. Ligation Reaction, DH10B Transformation, and Colony PCR

D-1. Ligation Reaction

1) Set up the ligation reaction as the follows

5x Ligase buffer	3.0 µl
pSiEB(Bbs1 digested)	1.0 µl
Assembled-Fragments	3.0 µl
ddH_2O	7.0 µl
T4 DNA Ligase	1.0 µl
Total	15 µl

2) Incubate reactions at room temperature for 30min.
3) Reduce background by cutting ligation products with Hind*III*:

Ligation product	7.5μl
10x CutSmart	10.0μl
ddH_2O	81.5μl
Hind*III*	1.0μl
Total	100μl

4) incubate at 37°C for 10~20min.
5) Perform ethanol precipitation. Let the pellet air dry, and dissolve it in 30ul ddH2O. To the remaining 7.5ul ligation product add 192.5ul ddH2O and perform ethanol precipitation. Let the pellet air dry, and dissolve it in 30ul ddH2O.

D-2. DH10B Transformation & Colony PCR

Please follow relevant MOLab regular protocols.

[**Optional**: DNA sequencing]

[**Optional**: Subcloning of the whole siRNA cassette into adenoviral vector system]

Appendix -1: How to Design Multiplex siRNA Fragments (for 3 to 5 siRNA Sites)

[NOTE: UPPER CASE indicates the top-strand sequence; lower case indicates the reverse-complement sequence of bottom strand]

IF THREE siRNAs ARE CONSTRUCTED

Fragment #1 (using pH1U6-T1) (x *BsaI*)

siRNA-A Fwd
aaaaaZZZZZZZZZZZZZZZZZZtttttAGAGTGGTCT

siRNA-A Fwd w/ *BsaI-BbsI-A*
ggtGGTCTCGggcaaaaaaZZZZZZZZZZZZZZZZZZ

siRNA-B Rev
aaaaayyyyyyyyyyyyyyyyyyttttTTCGTCCTTTC

siRNA-B Rev w/ *BsaI-B*
ggtGGTCTCG**cgtt**Aaaaaayyyyyyyyyyyyyyyyyy

Fragment #2 (using pH1U6-T2) (x *BsaI*)

siRNA-B Fwd w/ *BsaI-B* (Common use)
ggtGGTCTCGaacgTttttGTCTCATACAGAACTTATAA

siRNA-C Rev
aaaaaxxxxxxxxxxxxxxxxxxttttTTCGTCCTTT

siRNA-C Rev w/ *BsaI-BbsI-R*
ggtGGTCTCG**gcca**aaaaaxxxxxxxxxxxxxxxxxx

If FOUR siRNAs Are Constructed

Fragment #1 (using pH1U6-T1) (x *BsaI*)

siRNA-A Fwd
aaaaaZZZZZZZZZZZZZZZZZZtttttAGAGTGGTCT

siRNA-A Fwd w/ *BsaI-BbsI-A*
ggtGGTCTCGggcaaaaaaZZZZZZZZZZZZZZZZZZ

siRNA-B Rev
aaaaayyyyyyyyyyyyyyyyyyttttTTCGTCCTTTC

siRNA-B Rev w/ *BsaI-B*
ggtGGTCTCG**cgtt**Aaaaaayyyyyyyyyyyyyyyyyy

Fragment #2 (using pH1U6-T2) (x *BsaI*)

siRNA-B Fwd w/ *BsaI-B* (Common use)
ggtGGTCTCGaacgTttttt**GTCTCATACAGAAC**TTATAA

siRNA-C Rev
aaaaaxxxxxxxxxxxxxxxxxxxttttt**TTCGTCCTTT**

siRNA-C Rev w/ *BsaI-C*
ggtGGTCTCG**cgga**Aaaaaaxxxxxxxxxxxxxxxxxxx

Fragment #3 (using pH1U6-T2) (x *BsaI*)

siRNA-C Fwd w/ *BsaI-C*
ggtGGTCTCGtccgTttttt**GTCTCATACAGAAC**TTATAA

siRNA-D Rev
aaaaawwwwwwwwwwwwwwwwwwttttt**TTCGTCCTTT**

siRNA-D Rev w/ *BsaI-Bbs1-R*
ggtGGTCTCG**gcca**aaaaawwwwwwwwwwwwwwwwww

If FIVE siRNAs Are Constructed

Fragment #1 (using pH1U6-T1) (x BsaI)

siRNA-A Fwd
aaaaaZZZZZZZZZZZZZZZZZZZttttt**AGAGTGGTCT**

siRNA-A Fwd w/ BsaI-BbsI-A
ggtGGTCTCGggcaaaaaaZZZZZZZZZZZZZZZZZZZ

siRNA-B Rev
aaaaayyyyyyyyyyyyyyyyyyttttt**TTCGTCCTTTC**

siRNA-B Rev w/ BsaI-B
ggtGGTCTCGcgttAaaaaayyyyyyyyyyyyyyyyyyy

Fragment #2 (using pH1U6-T2) (x BsaI)

siRNA-B Fwd w/ BsaI-B (Common Use)
ggtGGTCTCGaacgTttttt**GTCTCATACAGAAC**TTATAA

siRNA-C Rev
aaaaaxxxxxxxxxxxxxxxxxxxttttt**TTCGTCCTTT**

siRNA-C Rev w/ BsaI-C
ggtGGTCTCGcggaaaaaaxxxxxxxxxxxxxxxxxxx

Fragment #3 (using pH1U6-T2) (x BsaI)

siRNA-C Fwd w/ BsaI-C (Common use)
ggtGGTCTCGtccgTttttt GTCTCATACAGAAC TTATAA

siRNA-D Rev
aaaaawwwwwwwwwwwwwwwwwttttt TTCGTCCTTT

siRNA-D Rev w/ BsaI-D
ggtGGTCTCGacgcaaaaawwwwwwwwwwwwwwwwww

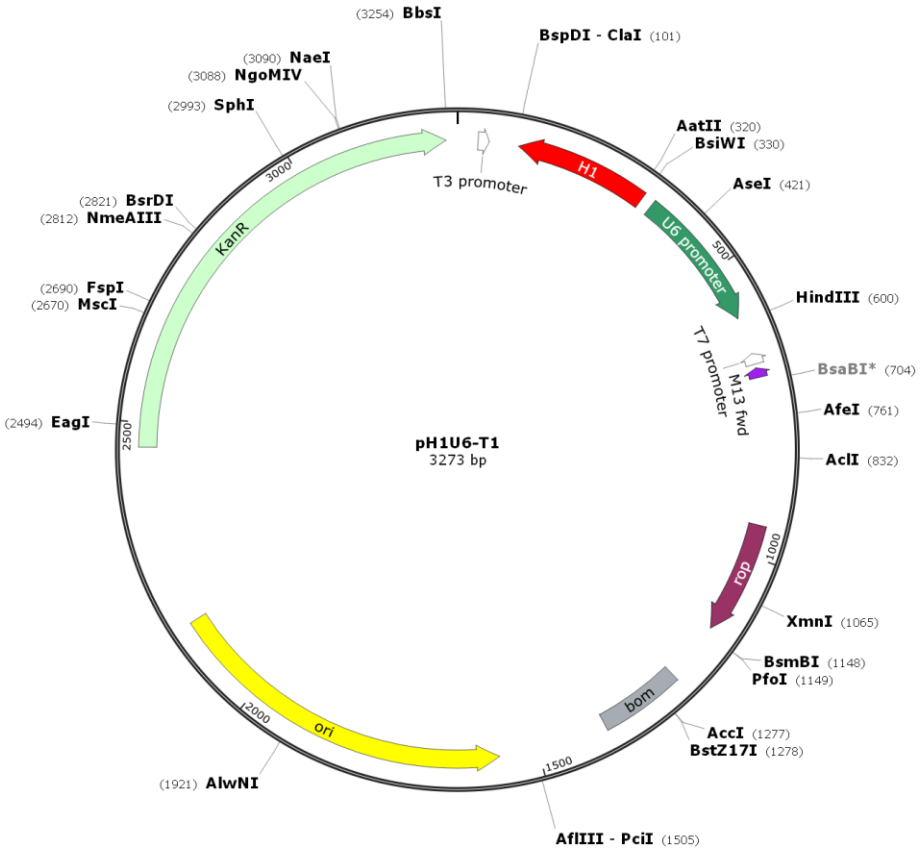
Fragment #4 (using pH1U6-T2) (x BsaI)

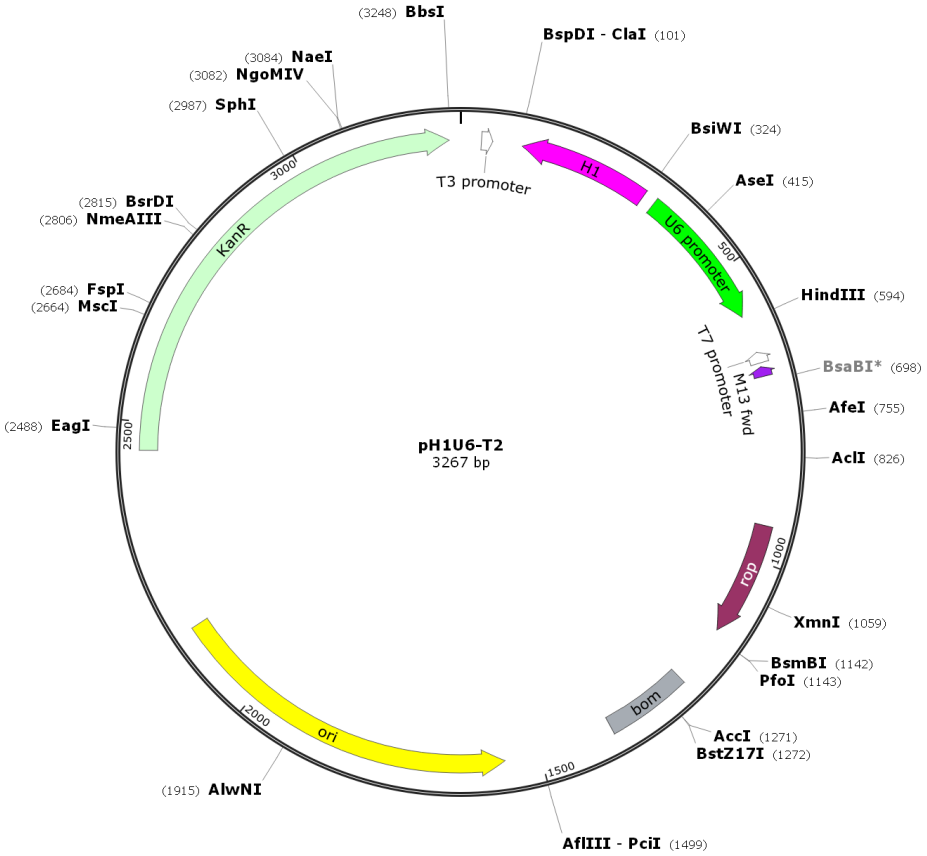
siRNA-D Fwd w/ BsaI-D (Common use)
ggtGGTCTCGgcgtTttttt GTCTCATACAGAAC TTATAA

siRNA-E Rev
aaaaavvvvvvvvvvvvvvvvvvttttt TTCGTCCTTT

siRNA-E Rev w/ BsaI-BbsI-R
ggtGGTCTCGgccaaaaaavvvvvvvvvvvvvvvvvvv

Appendix 2: FAMSi Template Vectors





Appendix 3: pSiEB Destination Vectors