Technology
Biomyx
Innovation & Convenience for Life Sciences

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pH1-siRNA

Catalog# P8020
Lot# On label

Materials Provided

1. pH1-siRNA plasmid: 20 μg in 40 μl TE (pH7.5), 0.5 mg/ml.
2. H1 Sequencing primer (5'-TGCATGTCGCTATGTGTTCT-3', nt 144-163): 0.5 nmole (3 μg), 10 pmole/μl (60 ng/μl).
3. Product Information Sheet.

Receiving and Storage:

Upon receiving, spin the vials briefly in a microcentrifuge to collect the contents. Store the products at 2-8^{0}C if used immediately and store at –20^{0}C for extended storage.

Prokaryotic selection:

Kan/Neo(782-1576): Neomycin resistance gene driven by the β-lactamase promoter (P_{bla}) confers Kanamycin resistance in bacteria.

Eukaryotic expression of siRNA:

H1 RNA Promoter (1-260): A RNA polymerase III promoter derived from human H1 RNA gene (X16612).

Eukaryotic selection:

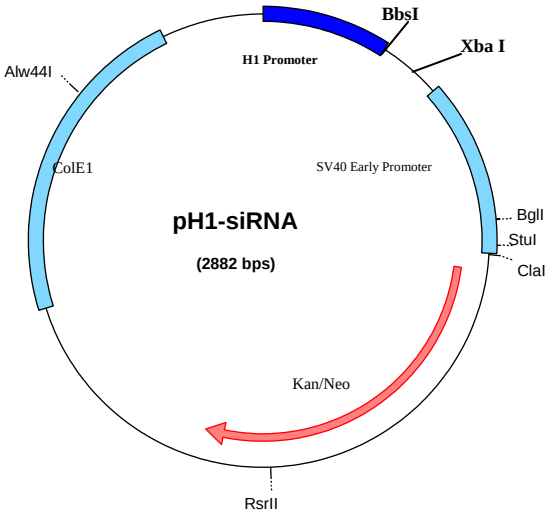
Kan/Neo(782-1576): Neomycin resistance gene driven by SV40 early promoter (390-746), with TK termination and polyadenylation signal(1761-2022), confers G418 resistance in eukaryotic cells.

Replication origin:

ColE1 origin (2023-2858): prokaryotic replication origin.

Circular Map of pH1-siRNA:

Go to www.biomyx.net for the full-length sequence of pH1-siRNA.



Promoter/MCS Junction Sequence:

```
                                          Xba I
5′-A CTC TTT CCC ATG GTC TTC ATC CTA TCT AGA CAT C-3′
3′-T GAG AAA GGG TAC CAG AAG TAG GAT AGA TCT GTA G-5′
                    Bbs I
```

CONSTRUCTION OF siRNA EXPRESSION VECTOR:

Short antisense and double-stranded RNAs of ~22 (bp) have been shown to suppress the expression of genes containing the same nucleotide sequence in mammalian cells. pH1-siRNA is designed for expressing short RNAs in mammalian cells. The short RNAs expressed can be single strand sense/antisense or double stranded siRNAs with a stem loop structure (hairpin). To do this, a DNA sequence that encoding the intended RNA has to be inserted into MCS region in pH1-siRNA. Here's a brief protocol for designing and cloning of siRNA-encoding oligos.

Target sequence selection: The first step in constructing a siRNA expression vector is to identify target sequences in the gene of interest. Although siRNA seems to be more effective for gene silencing than antisense DNA oligos, not all siRNAs selected will induce RNAi to the same extent. It was estimated that a little over half of the siRNAs give rise to over 50% reduction in target mRNA levels and about a quarter of siRNAs provide a 75-95% reduction. Therefore, the most important factor to a successful gene silencing with siRNA may be the selection of sequences. There does not seem to be generally applicable rules, unfortunately, for the selection of target sequences. Listed below are a few considerations when scanning the target gene for potential siRNA sites:

1. Select 3-4 equally spaced sites so that not all of them will fall into a non-accessible region along the target mRNA.
2. The termination signal for RNA polymerase III is "TTTTT". Hence, the target site should not have any continuous stretches of more than four "T"s.
3. It's best that target site starts with one or two "A" which corresponds to the native transcription start nucleotide in H1 RNA, and is complementary to the poly Us derived from the stop sequence (TTTTT) at the end of the hairpin.
4. After the selection of the target sequence, compare to an appropriate genome database to avoid nonspecific silencing of other genes of significant homology.

Loop selection: The loop structures in the middle of the hairpin have significant effects on the efficiency of siRNA export into the cytosol where it's perceived to exert its effect. In the hypothetical examples given below, the miR-23 loop (CUUCCUGUCA), shown to enhance the export of siRNA and processing into siRNA (Lagos-Quintana et al., 2001; Kawasaki and Taira, 2003)

Vector Construction:

One of the two approaches listed below can be used to clone siRNA-encoding sequences into the pH1-siRNA vector. The PCR approach (Approach B) was found to be more consistent than the direct insertion of dsDNA oligos when using pH1-siRNA.

Approach A: Direct Insertion of dsDNA oligo

Oligo design: Here's an example of oligo design for a target site (nt1968-1981) in human B-Raf gene (NM_004333). *The target sequence, both sense and antisense are underlined; letters in upper case at the ends are single strand overhangs for insertion into the linearized pH1-siRNA and those in the middle will form the loop of the hairpin; letters in italics are Pol III stop signal.*

Design oligos for the target sequences of your interest by replacing the underlined nucleotide sequence with the target sequence you wish to insert into the vector. Although not absolutely essential, it's best that the target sequence begins with a "A" as does the native H1 RNA.

Synthesized DNA oligos:

```
#1: 5′-CCCAgctttcagtcagatgtatatgcaCTTCCTGTCAtgcatatacatctgactgaaagcTTTTT-3′
#2: 5′-CTAGAAAAAgctttcagtcagatgtatatgcaTGACAGGAAGtgcatatacatctgactgaaagc-3′
```

Resulting annealed dsDNA oligo:

```
5′- CCCAgctttcagtcagatgtatatgcaCTTCCTGTCAtgcatatacatctgactgaaagcTTTTT    -3′
3′-    cgaaagtcagtctacatatacgtGAAGGACAGTacgtatatgtagactgactttcgAAAAGATC-5′
```

Expressed RNA product:

```
5′-    agcuuucagucagauguauaugcaCUUCC
3′-uuuucgaaagucagucuacauauacguACUGU
```

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Digestion of pH1-siRNA with BbsI and Xba I: The pH1-siRNA linearized with Xba I and Bbs I digestion will have the following ends, with the single strand overhangs underlined:

```
5′-A CTC TTT          CTAG ACA TC-3′
3′-T GAG AAA GGGT          TGT AG-5′
```

Set up the reaction in an Ependorf tube as follows: 2 µl NEB restriction buffer 2, 4 µl of pH1-siRNA (2 µg), 1 µl Xba I, 1 µl Bbs I and 12 µl of sterile DI water. Spin down the contents, mix well by pipeting up and down a few times. Incubate at 37^{0}C for 1-2 hours. Mix the reaction mixture with DNA loading dye and load onto a 1% Agarose gel. Cut out the band of the linearized pH1-siRNA (~2.9 kb) and recover the DNA with a gel extraction kit.

Annealing: Order the two oligonucleotides for the siRNA insert. Dissolve the oligonucleotides at a concentration of 1 µg/µl. Mix 2 µl of each oligonucleotide with 46 µl annealing buffer (100 mM K-acetate, 30 mM HEPES pH 7.4, and 2 mM Mg-acetate). Boil or heat the mixture at 90°C for 3 min, and then let it cool down gradually to room temperature within 30-60 min. The annealed oligos can be used directly for ligation or stored at -20°C.

Ligation: Bring the double-stranded siRNA insert to a concentration of 80 ng/µl (~10 pmole/L). Mix 1 µl of the insert (10 pmole) with 100-500 ng (0.05-0.25 pmole) of the linearized vector, 1 µl of 10X T4 DNA Ligase Buffer, 1 µl of T4 DNA Ligase. Bring the total volume with Nuclease-Free Water to 10 µl and incubate the ligation reaction at room temperature for 2 hours or over night. A negative control ligation could be performed with linearized vector alone and no insert. Then go to **Transformation of E. coli.**

Approach B: PCR amplification with phosphorylated primers: This approach uses phosphorylated primers containing siRNA-coding sequences to amplify the entire vector followed by self-ligation of the PCR product.

Oligo Design:

Design oligos for the target sequences of your interest by replacing the underlined nucleotide sequence with the lower strand of the target sequence (5'-3') you wish to insert into the vector.

Forward Primer:

5'-(P)tgtca **tgcatatacatctgactgaaagc** TTTTTGTCTTCATCCTATCTAGACAT-3'

Reverse Primer:

5'-(P)ggaag **tgcatatacatctgactgaaagc** GGGAAAGAGTGGTCTCATACA-3'

Cloning:

1. Phosphorylate the primers with T4 PNK. (You may skip this step if you ordered the primers 5'-phosphorylated). To an Ependorf tube, add 1 µl of T4 PNK (NEB), 1 µl of 10 x PNK buffer, 1 µl of 10 mM ATP, 200 pmole of the forward primer, 200 pmole of the reverse primer and appropriate amount of DI water to make a final volume of 20 µl. Mix well and incubate at 37^{0}C for 30 min. Use 1.25 µl of the mixture to the PCR reaction in step 2.
2. Amplify the vector with phosphorylated primers. (*Pfu from Stratagene is used here but other proof-reading DNA polymerases can be used as well)*: Assemble the PCR reaction by mixing the following components: 2.5 µl 10 x Cloned pfu buffer, 2 µl of dNTP (2.5 mM), 1.25 µl DMSO, 30 ng of pH1-siRNA, 1.25 µl of phosphorylated forward primer and reverse primer mixture from step 1, 1 µl pfu DNA polymerase, add DI water to 25 µl of total volume. Mix well and denature at 94^{0}C for 2min followed by 20 cycles of: 1' at 94^{0}C, 1' at 52^{0}C, and 4min at 72^{0}C with a final 10 min extension at 72^{0}C.
3. Digest with Dpn I (optional): Add 0.5ul Dpn I (NEB) into the PCR reaction, mix well and incubate for 1 hour at 37^{0}C. This step, although not necessary, increases the cloning efficiency .
4. Gel Purify the PCR product: Run all the samples on a 1% Agarose gel, cut out the PCR product band (~3.0 kb) and purify with a gel extraction kit (e.g. Qiagene Cat#28706).
5. Ligation: Use 1/3 of the purified PCR product, add appropriate amount of 10 x T4 DNA ligation buffer and 1 ul T4 DNA ligase, as described above for **Approach A**.

The resulting construct will express the following RNA:

5′-**Agcuuucagucagauguauaugca**CUUCCUGUCA**ugcauauacaucugacugaaagcU***UUUU***-3′

Transformation of *E. coli*: Use 1-3 µl of the ligation mixture to transform 50-100 ul of competent E. coli such as DH5-α, JM109 or XL1-Blue. Plate the transformants onto LB Agar plates containing 50µg/ml Kanamycin (Biomyx Cat# LC-1100) and incubate 14 to 16 hours at 37^{0}C. Pick 4-12 single colonies and grow up each colony in 1.5 ml LB plus 50 µg/ml Kanamycin liquid medium in a 37^{0}C shaker.

Minipreps of plasmid DNA: Extract the plasmid DNA from 2-6 of the grown up colonies with your method of choice such as a Qiaprep Spin Miniprep kit (Qiagene, 27104) according to the manufacturer's instructions.

Insert Confirmation: Digestion of the resulting plasmid DNA along with the parental pU6-siRNA (0.5 ug) with Xba I and Aat II can be used to confirm the presence of the insert. The insert should then be verified by DNA sequence analysis with the H1 sequencing primer supplied (Part# P8020B). This primer lies ~60 bp upstream to the insert (nt 144-163).

Maxiprep of plasmid DNA: After the insert is verified by DNA sequence analysis, the same colony can be grown up in 200-500 ml of LB plus 50 µg/ml Kanamycin liquid medium to prepare more plasmid DNAs for transfecting mammalian cells. Typically, ~0.5 mg of pH1-siRNA-derived plasmid can be obtained from a 200 ml overnight culture.

Further Readings:

Reviews

Zamore P.D. (2003). RNA interference: listening to the sound of silence. Nature Structural Biology, 8(9):746-750.
Ahlquist P. (2002). RNA-dependent RNA polymerases, viruses, and RNA silencing. *Science* **296**:1270-1273.
Fire, A. (1999) RNA-triggered gene silencing. Trends in Genetics, 15:358-363.
Ding, S.W. (2000) RNA silencing. Curr. Opin. Biotech. 11:152-156.
Gura, T. (2000) A silence that speaks volumes. *Nature* 404:804-808.
Hammond, S.M., Caudy, A.A., and Hannon, G. J. (2001) Post-Transcriptional Gene Silencing by Double-stranded RNA. *Nature Rev Genetics* **2**: 110-119.
Hannon GJ. (2002). RNA interference. *Nature* **418**:244-251.
Hunter CP. (2000) Shrinking the black box of RNAi. Current Biology 10:R137-R140.
Hutvagner G, Zamore PD. (2002) RNAi: nature abhors a double-strand. *Curr Opin Genet Dev* **12**:225-232.
McManus MT, Sharp PA. (2002) Gene silencing in mammals by small interfering RNAs. *Nat Rev Genet* **3(10)**:737-747.
Plasterk RHA. (2002). RNA silencing: the genome's immune system. *Science* **296**:1263-1265.
Tuschl T. (2002). Expanding small RNA interference. *Nature Biotechnology* **20**:446-448.

Original Research papers

Brummelkamp, TR, Bernards, R, and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**:550-553.
Elbashir, SM, Harborth J, Lendeckel W, Yalcin A, Weber K, and Tuschl T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494-498.
Gitlin L, Karelsky S, and Andino R. (2002). Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature* **418**:430-434.
Kawasaki, H., and Taira, K., 2003. Short hairpin type of dsRNAs that are controlled by tRNA^{val} promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells. Nuc Acids Res., 31(2): 700-707
Lagos-Quintana, M., Rauhut R., Yalcin, A., Meyer, J., Lendeckel, W and Tuschl, T. 2001, Identification of novel genes coding for small expressed RNAs. Science. 294, 853-858
Martinez J, Patkaniowska A, Urlaub H, Luhrmann R, Tuschl T. (2002).Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell. 110(5):563-74.
McCaffrey AP, Meuse L, Pham T-T, Conklin DS, Hannon GJ, and Kay MA. (2002). RNA interference in adult mice. *Nature* **418**:38-39.
Miyagishi, M, and Taira, K. (2002). U6-promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. *Nature Biotechnol* **19**:497-500.
Paddison PJ, Caudy AA, Bernstein E, Hannon GJ and Conklin DS. (2002). Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes & Development* **16**:948-958.
Paddison PJ, Caudy AA, Hannon GJ. (2002) Stable suppression of gene expression by RNAi in mammalian cells. *Proc Natl Acad Sci USA* **99(3)**:1443-1448.
Paul, CP, Good, PD, Winer, I, and Engelke, DR. (2002). Effective expression of small interfering RNA in human cells. *Nature Biotechnol* **19**:505-508.
Sui G, Soohoo C, Affar EB, Gay F, Shi Y, Forrester WC, and Shi Y. (2002) A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc Natl Acad Sci USA* **99(8)**: 5515-5520.
Wianny F, Zernicka-Goetz M. (2000) Specific interference with gene function by double-stranded RNA in early mouse development. *Nat Cell Biol* **2**:70-75.