Technology
Biomyx
Innovation & Convenience for Life Sciences

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pU6-siRNA

Catalog# P8018
Lot# On label

Materials Provided

1. pU6-siRNA plasmid: 20 μg in 40 μl TE (pH7.5), 0.5 mg/ml.
2. U6 sequencing primer (5'-CACAAAAGGAAACTCACCCT-3', nt239-258): 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:

U6 RNA Promoter (1-319): A RNA polymerase III promoter derived from murine U6 snRNA gene (X06980).

Eukaryotic selection:

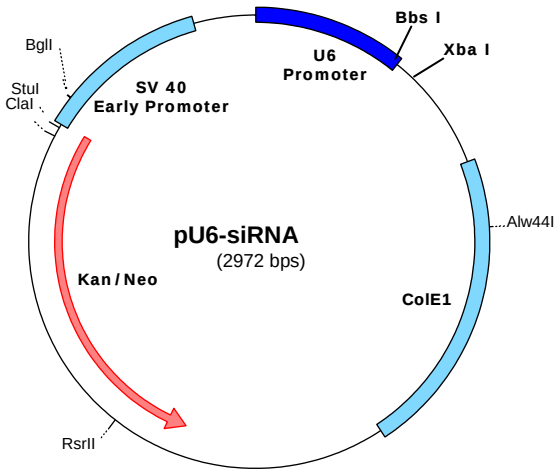
Kan/Neo (1656-2490): Neomycin resistance gene driven by SV40 early promoter (2486-2842), with TK termination and polyadenylation signal (1210-1471), confers G418 resistance in eukaryotic cells.

Replication origin:

ColE1 origin (328-1209): prokaryotic replication origin.

Circular Map of pU6-siRNA:

Go to www.biomyx.net for the full-length sequence of pU6-siRNA.



Promoter/MCS Junction Sequence:

```
                                                  Xba I
5′-A GCC TTG TTT GTG GTC TTC ATC CTA TCT AGA CAT A-3′
3′-T CGG AAC AAA CAC CAG AAG TAG GAT AGA TCT GTA T-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. pU6-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 pU6-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 work. 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 the target sequences, for which there does not seem to be generally applicable rules, unfortunately. 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.
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 at least one "G" which corresponds to the native transcription start nucleotide in U6 RNA.
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 loops in the middle of the hairpin structure 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 (Lagos-Quintana et al., 2001; Kawasaki and Taira, 2003)

Vector Construction:

One of the two approaches can be used to clone siRNA-encoding sequences into the pU6-siRNA vector.

Approach A: Insertion of dsDNA oligo:

Oligo design: Here's an example of oligo design for a target site (nt1969-1982) 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 pU6-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 "G" as does the native U6 RNA.

Synthesized DNA oligos:

```
#1: 5′-TTTGctttcagtcagatgtatatgcatTTCCTGTCAatgcatatacatctgactgaaagcTTTTT-3′
#2: 5′-CTAGAAAAAgctttcagtcagatgtatatgcatTGACAGGAAGatgcatatacatctgactgaaag-3′
```

Resulting annealed dsDNA oligo:

```
5′- TTTGctttcagtcagatgtatatgcaCTTCCTGTCAtgcatatacatctgactgaaagcTTTTT    -3′
3′-    gaaagtcagtctacatatacgtGAAGGACAGTacgtatatgtagactgactttcgAAAAAGATC-5′
```

Expressed RNA:

```
5′-    gcuuucagucagauguauaugcauCUUCC
3′-uuuucgaaagucagucuacauauacguaACUGU
```

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Digestion of pU6-siRNA with BbsI and Xba I: The pU6-siRNA linearized with Xba I and Bbs I digestion will have the following ends, with the single strand overhangs underlined:

```
5′-A GCC TTG          CT AGA CAT A-3′
3′-T CGG AAC AAAC           T GTA T-5′
```

Set up the reaction in an Ependorf tube as follows: 2 μl NEB restriction buffer 2, 4 μl of pU6-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 pipetting 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 pU6-siRNA (~2.9 kb) and recover the DNA with a gel extraction kit.

Annealing of oligos: 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. The annealed oligos can be used directly in a ligation reaction or stored at -20°C.

Ligation: Bring the double-stranded siRNA insert to a concentration of 80 ng/μl (~10 pmole/μl). 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. Then use Nuclease-Free Water to adjust the total volume to 10 μl and incubate the ligation reaction at room temperature from 2 hours or overnight. *A negative control ligation could be performed with linearized vector alone*. 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 **atgcatatacatctgactgaaagc** TTTTT GTCTTCATCCTATCTAGACAT-3'
Reverse Primer:
5'-(P)ggaag **atgcatatacatctgactgaaagc** AAACAAGGCTTTTCTCCAAGG -3'

Cloning:

1. Phosphorylate the primers: *(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 for 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 pU6-siRNA, 1.25 μl of phosphorylated forward primer and reverse primer mixture from step 1, 1 μl pfu DNA polymerase, add DI water to make the total volume to 25 μl. 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. Qiagen Cat#28706).
5. Ligation: Use 1/3 of the purified PCR product, add appropriate amount of 10 x T4 DNA ligation buffer and 1 μl T4 DNA ligase, as described above for **Approach A**. *The resulting construct will express the following RNA product:*

5′-**gcuuucagucagauguauaugcau**CUUCCUGUCA**augcauauacaucugacugaaagc***UUUUU-3′*

Transformation of *E. coli*: Use 1-3 μl of the ligation mixture to transform 50-100 ul of competent E. coli cells such as DH5-α, JM109 or XL1-Blue. Plate the transformants onto a LB Agar plate containing 50 μg/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.

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

Insert Confirmation: Digestion of the resulting plasmid DNA along with the parental pU6-siRNA (0.5 ug) with Xba I and Pvu II can be used to confirm the presence of the insert. The insert should then be verified by DNA sequence analysis with the U6 sequencing primer supplied (Part# P8020B). This primer lies ~60 bp upstream to the insert (nt239-258).

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 make more plasmid DNAs for transfecting mammalian cells. Typically, ~0.5 mg of pU6-siRNA-derived plasmid can be obtained from a 200 ml overnight culture.

Further Readings:

Review

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