

Cloning siRNA duplexes into pSUPER or pSUPERretro vectors

Design considerations

1) Oligos should be designed with BamHI (or BglII) and XhoI overhangs.

Note: the protocol from Oligo Engine calls for designing BamHI and HindIII overhangs for cloning into the BglII and HindIII sites of the pSUPER vectors. However, these sites are right next to each other and it is impossible to get much cutting by the second enzyme once the first enzyme has cut. This results in an extremely high background. As a result of this problem, the vendor's protocol calls for phosphatase treating the vector to decrease this background. In this case, the oligos would then have to be phosphorylated in order for the ligation to work. This results in a much poorer cloning efficiency because 1) phosphatase treatment tends to damage a fair number of vector ends, 2) phosphorylation of the oligos is not 100% efficient, and 3) there is a high amount of single cut vector which covalently attaches to oligos but cannot circularize and form colonies. In addition, this procedure results in a number of extra steps which are not required if the oligos are simply cloned directly into the BglII and XhoI sites.

2) We typically use unpurified oligos (200nMole scale) from either Oligoengine or from Integrated DNA technologies. Using our protocol, unpurified oligos typically work reasonably well. We have found that when using purified oligos, more colonies can be obtained but we are not convinced that you get that many more full length inserts relative to mutant or truncated inserts (we think this is because the cloning procedure selects for only those double stranded oligos that are full length - i.e. oligos that are not full length generally will not clone).

3) It has more recently been found that secondary structure plays a role in accessibility of the mRNA to siRNAs (although early reports suggested the contrary). The siRNA design program from OligoEngine carries out a folding analysis and provides data on the predicted accessibility of siRNAs and this is likely helpful for identifying good candidate target sites. There have also been rumblings that lower G/C have a greater chance of working (perhaps related to the above consideration) so we typically set these parameters from 30% to 50% G/C.

4) There are also reports out now indicating that off site targeting is an important consideration in the design of siRNAs. Gene chip analysis shows that as little as 12bp homology can in some cases result in suppression. In addition, we have noted significant toxicity unrelated to specific targeting with some of our earlier siRNAs and this is likely due to off site targeting. Therefore, a blasting program which picks up these homologies is important for selecting targets that have the least chance of off site targeting. The Oligoengine Workstation takes this issue into consideration. In addition, their program uses the UniGene database so you don't have to sort through numerous different sequence versions of the same gene in carrying out your analysis.

Protocol

- 1) Spin dried oligos in microfuge for 15-30 seconds (high speed) to make sure pellet is on bottom.
- 2) Add 150ul TE and let sit for 15 minutes.
- 3) Suspend using a filtered pipette tip.
- 4) Quantitate oligos

Annealing

25ug Oligo 1

25ug Oligo 2

10ul 10X MRB

Add Ultrapure H₂O up to a volume of 100ul (final concentration = 0.5ug/ul)

- 5) Vortex
- 6) Spin down briefly
- 7) Put eppendorf tube in a rack in a 68°C water bath. We usually put a container in 68°C water bath and let it fill with the 68°C water, then we put the rack with the tube in the water that is in the container. Keep tube at 68°C for 10 minutes.
- 8) After 10 minutes at 68°C, take container with water and tube out of water bath and let cool slowly until it reaches room temperature.
- 9) Keep this stock frozen but also make a dilution to a working concentration of 10ng/ul in 1X MRB (10ul annealed oligos + 50ul 10X MRB + 440ul H₂O).

Cloning

Use 2ul of diluted (10ng/ul) annealed oligos for a standard 20ul ligation reaction. It is also helpful to add 1ul T4 polynucleotide kinase to ligation reaction (no other modifications to the ligation reaction are required since T4 polynucleotide kinase works well in ligase buffer).

Notes on cloning:

- 1) It is helpful to carry out a control ligation in which vector but no oligo is added to the ligation reaction. Typically, you should get at least 2 fold more colonies in ligations containing oligo compared to ligations with vector alone (using crude unpurified oligos). If using HPLC or gel purified oligos, you should expect at least 10 - 500 fold more colonies.
- 2) We typically use the low melt ligation protocol from the eZclone Systems web site (www.ezclonesystems.com) because we find that once you get used to it, it is very easy, fast, and efficient. In addition, the vector digestion can be scaled up and the agarose plug can be kept at -20C (and it can be frozen and thawed a number of times).
- 3) This cloning procedure is straight forward and normal low efficiency competent cells should work well [this protocol (Calcium Chloride Method) also listed on the flemingtonlab.com web site].

THE FLEMINGTON LAB: Tulane Health Sciences Center, New Orleans, LA 70112

www.flemingtonlab.com, Tel: 504-988-1167, email: eflemin@tulane.edu

Diagnostics

- We typically first carry out a diagnostic digest to select clones that have an insert using EcoRI and XhoI. You must run EcoRI/XhoI digested vector on the same gel to make sure you can detect those clones that have an insert (i.e. look for a size difference).
- After we have at least two mini preps with inserts, we will have them sequenced using either the T3 primer for pSUPER or the pSUPERretro1 primer (5'-ATCCAGCCCTCACTCCTT-3') for pSUPERretro.

Note: we get good yields of high quality DNA using the Boil prep method listed on the Flemington Lab web site. In addition, once positive clones have been identified, the RNA can be digested and these minis will then typically give very good sequence results (mini preps should be digested with 100ug RNase in the presence 300ul 1X MRB for 1 hr, phenol extracted two times (300ul), 30 ul of 3M NaOAc and 800ul of 100% EtOH are added, the tubes are vortexed and spun at high speed in a microfuge for 30min. The samples should be washed one time with 800ul 70% EtOH, dried briefly, and resuspended in 20ul TE for sequencing).

Final Note: we are assembling a list of siRNA vectors to be put on our web site to foster cooperation and possibly collaborations. If you generate an siRNA that works well for a specific gene, we will list it on our web site (along with an experiment showing that it works). We will not list the target site but if someone inquires about your siRNA, we will put that person in contact with you to discuss possible collaborations.

Solutions

10X Medium Restriction Buffer (10XMRB)

100 mM Tris-Cl (pH 7.5)
500 mM NaCl
100mM MgCl
10mM DTT

10ml of 10X MRB

1ml 1M Tris (pH 7.5)
1ml 5M NaCl
1ml 1M MgCl₂
100ul 1M DTT