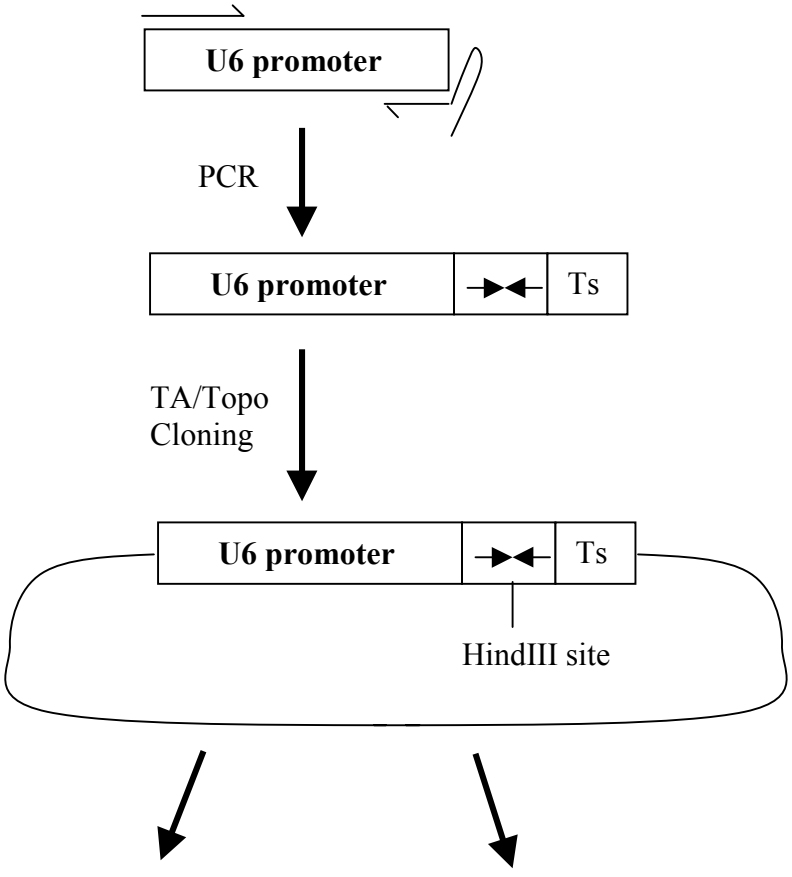
A PCR-based strategy for cloning short hairpin sequences: "PCR shagging".

Our overall approach is to use an RNA polymerase III promoter to drive expression of encoded short hairpin RNA (shRNA). For this purpose we use the U6 snRNA promoter and maintain the transcript initiating "G" nucleotide of the U6snRNA transcript. There by, hairpin sequences will start with a "G". Termination is mediated by a run of Ts at the end of the hairpin.

The major difference between our hairpins those reported by others is that we went through a battery of tests of hairpin length and structure, and found that hairpins of 27 to 29nt in length are more effective than hairpins of 19nt and 21nt. Additionally, we use a few G-U pairing in the hairpin stem (which are permitted in dsRNA alpha helices) to stabilize hairpins during propogation in bacteria.

We have developed a fast and effective, PCR-based strategy to clone shRNA sequences. In this strategy, short hairpin RNA (shRNA) sequences are converted into a single ~73nt primer sequences onto which are added 21nt of homology to the human U6 snRNA promoter. Such primers have performed flawlessly so far in PCR reactions (n>40) and subsequent cloning.



system)

There are several steps in generating hairpin primers. First, a 29nt "sense" sequence which ends with a "C" is picked out from the coding sequence of gene of interest. Second, the actual hairpin is constructed in a 5'->3 orientation with respect to the intended transcript.

```
        Anti-sense                Loop              Sense                Term
ggctatgaagagatacgccctggttccGaagcttGggaaccagggcgtatctcttcatagccTTTTTTG
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Predicted shRNA structure

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                5'->3' Anti-sense strand
       -------|                          GAA
              GGCUAUGAAGAGAUACGCCCUGGUUCC   G
              CCGAUACUUCUCUAUGCGGGACCAAGG   C
          UU^                           GUU
                3'<-5' Sense strand
```

Third, a few stem pairing are changed to G-U by altering the sense strand sequence. **G-U base pairing seems to be essential for stability of short hairpins in bacteria** and does not interfere with silencing. Finally, the hairpin construct is converted to its "reverse complement" onto which is added 21nt of homology to the Human U6 promoter.

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Hairpin portion of the primer (~69nt)
CAAAAAAggctatgaagagaCacgccctgAttccCaagcttCggaaccagggcgtatctcttcatagcc
                                +
U6 promoter reverse primer sequence
Ggt gtt tcg tcc ttt cca caa

Final primer (5'->3', just as it would be ordered)
CAAAAAAggctatgaagagaCacgccctgAttccCaagcttCggaaccagggcgtatctcttcatagcc
Ggt gtt tcg tcc ttt cca caa
```

All of the aforementioned steps are automated using a program developed by Ravi Sachidanandam and Jeremiah Faith (CSHL) where either accession numbers from GenBank or raw sequences are required to generate hairpin PCR primers.

[Note: Don't let the G-U pairings represented in the primer fool you into thinking the primer is incorrect.]

A link to the hairpin primer generation program, the "RNAi oligo retriever", can be found at:

www.cshl.org/public/SCIENCE/hannon.html

Make sure that you enter accession numbers and sequences which match cDNA or exon sequences!

THE PROTOCOL

Ordering Primers

Since very little primer is required for the PCR reaction they can be ordered .05µmol scale from Sigma-Genosys or whomever. We find that PAGE purification is costly and unnecessary (PCR will fill in shorted primers!).

PCR

We use a pGEM1 plasmid containing the human U6 locus (N. Hernandez, CSHL) as the template for the PCR reaction. This vector contains ~500bp of upstream U6 promoter sequence. Since an SP6 sequence flanks the upstream portion of the U6 promoter, we use an SP6 oligo as the universal primer in U6-hairpin PCR reactions.

SP6 sequence: GATTTAGGTGACACTATAG

We have had consistently good results using **Taq polymerase** for PCR with **4% DMSO** and 50pmoles of each primers. (**For pENTR/D-Topo cloning [see below], I add .1uL of Vent to polish the ends.**)

PCR conditions: 95° for 3 min; 30 cycles of 95° for 30 sec, 55° for 30 sec, & 72° for 1 min; followed by one cycle of 72° for 10 min.

The PCR product will be ~600bp in length.

CLONING

We currently use two cloning technologies available from Invitrogen: T-A and directional topoisomerase-mediated cloning kits (catalog #K2040-10, K2400-20). The directional cloning kit is designed for Invitrogen's Gateway system. We use both kits according to the manufacture's instructions. **If using Topo-cloning, do NOT gel purify PCR products – it reduces the efficiency of the Topo-reaction.**

pENTR/D-Topo SP6 primer: **CACC** GATTTAGGTGACACTATAG

For convenient identification of clones containing the proper insert (20-100% for Topo-cloning), a HindIII site has been designed into the loop of the hairpin. A second HindIII site exists 5' of U6 promoter. Digesting clones with HindIII releases a ~500bp fragment.

SP6-U6 promoter PCR product sequence (with out hairpin)*.

SP6—HindIII—BamHI—U6 promoter→
gatttaggtgacactatagaatacAAGCTTGGCTGCAGGTCGACGGATCCCCCCGAGTCC
AACACCCGTGGGAATCCCATGG**NN**ACCATGGCCCCTCGCTCCAAAAATGCT
TTCGCGTCTCGCAGACACTGCTCGGTAGTTTCGGGGATCAGCGTTTGAGTA
AGAGCCCGCGTCTGAACCCTCCGCGCCGCCCCGG**N**CCCAGTGGAAAGAC
GCGCAGGCAAAACGCACCACGTGACGGAGCGTGACCGCGCGCCGAGCGC
GCGCCaaggtcgggcaggaagagggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagagagata
attagaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaaagtaataatttcttgggtagtttgcagttttta
aaattatgttttaaaatggactatcatatgcttaccgtaacttgaaagtatttcgatttcttggctttatatatcttgtggaaaggacgaaa
cacc[hairpin starts here with a "G"]

Lower case = plasmid or U6 Genbank sequence
Upper case = U6 promoter sequence not included in the Genbank sequence.
Note: "N"= ambiguous sequencing read.

*This sequence is subject to minor changes upon further sequence confirmation.

Good Luck using short hairpin activated gene silencing (ie SHAGGING)!
Please feel free to e-mail me questions concerning PCR-Shagging.

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