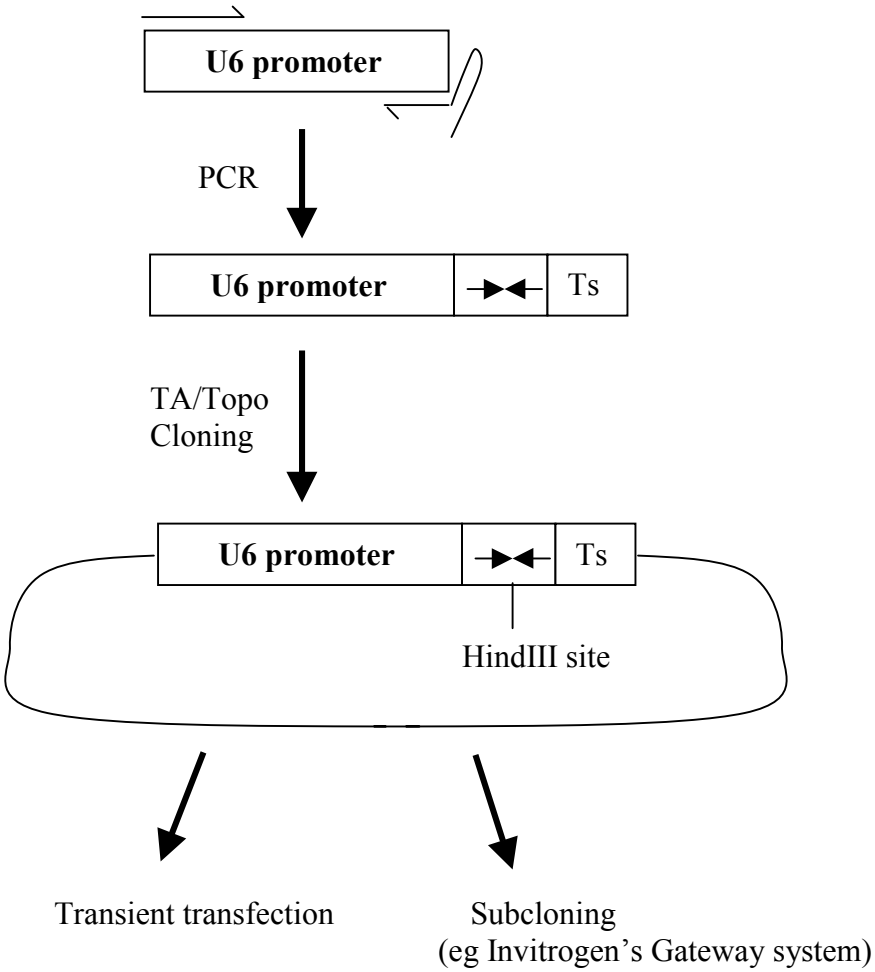
A PCR-based strategy for cloning short hairpin sequences: "PCR SHAGging".

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 human U6 snRNA promoter, maintaining 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.

We have found hairpins of 27 to 29nt in length to be as effective, if not more so, than hairpins containing 19nt and 21nt stems. Thereby**, our hairpins currently contain 29nt of stem**. An additional design feature is the inclusion of a few G-U pairings in the hairpin stem (which are permitted in dsRNA alpha helices) to stabilize hairpins during propagation in bacteria. *We are continuing to test length and structural features of our hairpins and will up date our web site accordingly*.

To quickly and efficiently generate hairpin constructs, we now employ a PCR-based strategy to clone shRNA sequences. In this strategy, short hairpin RNA (shRNA) sequences are converted into a single ~72nt primer sequence onto which are added 21nt of homology to the human U6 snRNA promoter.



So far, PCR hairpin primers have performed flawlessly in PCR reactions (n~100) and subsequent cloning. Of the bacterial clones which digest properly (20-100%) less than 25% contain sequence alterations (about the same as with ligation-SHAGging).

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

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ggctatgaagagatacgccctggttccGaagcttGggaaccagggcgtatctcttcatagccTTTTTT
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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 (~72nt)
AAAAAAggctatgaagagaCacgccctgAttccCaagcttCggaaccagggcgtatctcttcatagcc
                              +
U6 promoter reverse primer sequence
Ggt gtt tcg tcc ttt cca caa

Final primer (5'->3', just as it would be ordered)
AAAAAAggctatgaagagaCacgccctgAttccCaagcttCggaaccagggcgtatctcttcatagcc
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 can be used 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 or 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 at .05µmol scale from Sigma-Genosys or whomever. We find PAGE purification to be costly and unnecessary.

PCR

We use a pGEM1 plasmid (Promega) 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 primer. (For pENTR/D-Topo cloning [see below], I add .1uL of Vent to polish the ends – though this may not be necessary.)

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.

*Note: This second HindIII site is only found in the pGEMU6 shRNA PCR insert and is not available in pSHAG1, which is used for conventional cloning of hairpins (see web site).

Once the hairpin has been cloned into pENTR/D-topo, Invitrogen's Gateway system can be employed to quickly move the U6-shRNA insert to any vector. The *in vitro* clonase reaction is fast and robust at moving hairpins to recipient vectors (eg pBABE-puro).

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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