A protocol on how to clone shRNAs into pSicoR vectors.
Methods:

CLONING SMALL HAIRPINS INTO LENTIVIRAL VECTORS

There are many ways to clone small hairpin RNAs (shRNAs), and many of the methods depend on the vectors you are using.

Our lab often clones shRNA in pSicoR-derived vectors. Our most commonly used one has cloning sites downstream of a mouse U6 promoter, A 5 BstXI site and a 3 XhoI or BamHI site.

In order to clone shRNAs in pSicoR-vectors, we prepare linearized vector by digesting with the appropriate restriction enzymes followed by gel extraction. The insert shRNAs consist of two oligos that are complementary, and when annealed together contain the appropriate overhangs to allow cloning into the vector. Then, these two components are ligated together and transformed into competent bacteria.
Note: it is not necessary to phosphorylate the oligos, nor to order these PAGE or HPLC purified. This will just cost a lot of $$ and really is not necessary as long as the vector used has incompatible overhangs that are phosphorylated.

Design shRNA: There are many different programs that can help design shRNAs. A quick google search may help identify these. Whatever program you may use, we make use the following loop-sequence: TTCAAGAGA and a terminator consisting of 5 Ts. Additionally we start all our shRNAs with a G. The 3 arm is the targeting strand and the 5arm is 100% complementary to the targeting strand. We tend to clone ~5-10 shRNA per gene and test which ones work best.

An example:

GTATGCAGAGGATGAAAGATTAATTCTAGAGATTAATCTTTCATCCTCTGCATACTTTT
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Annealing Step: in a single tube, mix the following:
23 µl annealing buffer (10 mM Tris pH 7.5 to 8, 50 mM Sodium chloride, and 1 mM EDTA)
1 µl sense oligo (100µM)
1 µl antisense oligo (100µM)
Incubate the reaction for 5 minutes at 95°C and let the oligos gradually anneal to RT. This is done easiest in a PCR machine. Afterwards, the annealed oligos can then be placed on ice (or stored at minus 20°C) until used in the ligation step.
This is the annealed oligo stock; prior to use, it will be necessary to prepare a 1:20 dilution.

Ligation Step: in a single tube, mix in the following order
x µl ddH_2O
~ 50ng digested gel-extracted phosphorylated vector (IF dephosphorylation of your vector is necessary because you observe a high background of re-ligated vector or since blunt cloning is required: make sure that the oligos are phosphorylated)
1 µl of 1:20 diluted annealed oligos
1 µl 10X ligase buffer
1 µl ligase
10 µl total reaction volume
Incubate the above reaction at room temperature for 1-4 hours (or even better: overnight) and transform part of the ligation reaction into competent cells.

Miniprep and sequencing: Pick several colonies and perform minipreps on these. Sequence with an appropriate sequencing primer. In general, many shRNAs sequence just fine (if you send of your sequencing reactions to a commercial company, mention that these are hairpins!!!), although the signal drops significantly at the hairpin. If sequencing is very problematic you can try 3 different things:
1. digest the product with a restriction enzyme that can cut in the loop (if present) and sequence from both ends.
2. PCR the shRNA insert with two primers, and sequence these products. When the shRNA is not present in a coiled vector backbone, the sequencing tends to be a bit more efficient
3. Sequence a more concentrated vector prep.

Good luck!!

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