Identification of stem loop sequences:

We follow the design criterion described by Tom Tuschl. This is available at:
http://www.mpibpc.gwdg.de/abteilungen/100/105/sirna.html

Another useful site is provided by Greg Hannon:
http://katahdin.cshl.org:9331/RNAi/

In short we search for target sequences located throughout the mRNA. The consensus sequence should correspond to: $AAGN_{18}TT$. A 5' guanine is required due to the constraints of the U6 promoter. We recommend testing 4-5 targets for each gene of interest. As lentivirus pseudotyped with VSV-G is capable of infecting human cells we strongly recommend BLAST searching the GN_{18} sequences to both verify that your sequence is unique and to determine if there are potential human targets.

Oligo Design:
We have engineered a multiple cloning site immediately following the U6 promoter. An HpaI site leaves a blunt end prior to the –1 position in the promoter. The oligo design must incorporate a 5' T in order to reconstitute the –1 nucleotide of U6. An XhoI site cuts downstream of the U6 start site.

Oligo format:
Sense oligo: 5'T-(GN18)-(TTCAAGAGA)-(81NC)-TTTTTTC
Antisense oligo: Compliment of sense but with additional nucleotides at 5' end to generate XhoI overhang.

The loop sequence (TTCAAGAGA) is based upon Brummelkamp et al. (Science 2002).

We order our oligos through IDT with 5' phosphates and PAGE purified.

Cloning:
Oligos are resuspended in water at 60pmol/λ.

Annealing oligos:
1λ Sense oligo
1λ Antisense oligo
48λ Annealing Buffer

Annealing Buffer:
100mM K-acetate
30mM HEPES-KOH pH 7.4
2mM Mg-acetate

Incubate at 95° 4min
70° 10min
Decrease temperature to 4° slowly (.1°C/min)

Incubate at 4° 10 min

Digestion of pLentiLox 3.7
Digest 1-2μg with XhoI and HpaI
Treat with SAP or with CIP
Purify linearized fragment
Estimate concentration

Ligation
Ligate linearized product and annealed oligos at equimolar concentration. I typically use 60fmol of each component in a final concentration of 10μL.

Transformation:
I strongly recommend the use of an endA$^-$ strain of E. coli. We have had success with STBL-2 cells.

Testing clones:
We have had success testing for insertion of the stem-loop sequence with both colony pcr or by restriction digest. Insertion of insert causes a band shift of ~60bp in an XbaI/NotI fragment when compared to parental vector. This can be seen by 2% agarose gel electrophoresis. The following primer which corresponds to FLAP can be used to sequence into the U6 promoter and stem loop:
5'-cagtgcaggggaaagaatagtagac-3'.

Preparation of DNA:
We recommend the use of Qiagen Endo-Free Maxiprep Kits for all plasmids used in transfection of 293.T cells.