RNAi FOR MAMMALIAN CELLS
(Silencer siRNA Construction Kit)

Materials/Reagents:
Silencer siRNA construction kit (Ambion cat #1620)
DNA oligos, sense and antisense. Design 4-6 sets for each target gene.
Oligofectamine transfection reagent (Invitrogen cat # 12252-011)
Cells, standard tissue culture supplies

Procedure:

Step 1: Design and order DNA oligos:
- -Look for the first "AA" 75 nucleotides downstream of the transcription start site
- -Include the following 19 additional nucleotides
- -Verify that the GC content is between 30-70 percent (lower may be better)
- -Perform a BLAST search to verify sequence specificity to target gene
- -Determine sense and antisense DNA sequences and add 8 nt leader sequence "CCTGTCTC" to 3' end according to the example below

(There is also a tool on the Ambion website that will do this for you)

Example:
Target cDNA sequence: 5' AACGATTGACAGCAGCGGATTGCC 3'

Antisense siRNA template: 5'AACGATTGACAGCGGATTGCC**CCTGTCTC** 3'
Sense siRNA template: 5' AAGGCAATCCGCTGTCAATCG**CCTGTCTC** 3'

Step 2: Resuspend oligos:
Resuspend oligos to approximately 200 μM in nuclease-free dH20.
Dilute to 100 μM stocks.

Step 3: Hybridization and fill-in
Mix (in separate tubes for sense and antisense):

Sense reaction

T7 promoter primer	2 μl
DNA hybridization buffer	6 μl
Sense template	2 μl
	10 μl

Antisense reaction

T7 promoter primer	2 μl
DNA hybridization buffer	6 μl

Antisense template 2 μl
10 μl

heat to 70 C/ 5 min
room temp/ 5 min

Add to each reaction:

10X Klenow buffer	2 μl
10X dNTP mix	2 μl
nuclease-free dH20	4 μl
Klenow	2 μl

*should now have 20 μl total volume per tube

Mix, then incubate at 37 C for 30 min
Store at –20 C or use directly

Step 4: dsRNA synthesis

Mix (keep sense and antisense reactions separate):

Sense reaction

Sense template	2 μl
Nuclease-free dH20	4 μl
2X NTP mix	10 μl
10X T7 buffer	2 μl
T7	2 μl

Antisense reaction

Anti-sense template	2 μl
Nuclease-free dH20	4 μl
2X NTP mix	10 μl
10X T7 buffer	2 μl
T7	2 μl

Incubate for 2 hours at 37 C.
Combine sense and antisense reactions.

Incubate at 37 C overnight.

Step 5: siRNA purification

To the ds RNA, add:

Digestion buffer	6 µl
Nuclease-free dH20	48.5 µl
RNase	3 µl
DNase	2.5 µl

Incubate 2 hours at 37 C.

Add 400 µl siRNA binding buffer.
Incubate 2-5 min at room temp.

(Preheat 100 µl/reaction dH20 to 75 C)

Prewet filter cartridge with 100 µl siRNA wash buffer.
Bind siRNA by adding to column and spinning at 10,000 rpm for 1 min.
Wash with 2X 500 µl wash buffer.
Elute in 100 µl 75 C dH20.

Quantitate siRNA and store at –80 C until ready to use.
Dilute to 20 µM for siRNA transfection.

Step 6: siRNA transfection with Oligofectamine in 6 well plates
On the day before transfection, cells should be split into antibiotic-free media.
Cells should be 50% confluent on the day of transfection.

For each well of a 6 well plate:
Tube 1: Mix 12 µl of 20 µM siRNA with 200 µl Opti-MEM (or serum free media)
Tube 2: Mix 12 µl of Oligofectamine with 48 µl Opti-MEM in a separate tube
Incubate 7 min at room temp
Combine tubes 1 and 2, mix by inversion (important), and incubate for 20 min at room temp.
Add 128 µl fresh Opti-MEM for a final volume of 400 µl.
Add 400 µl of siRNA/Oligofectamine mix to the cells, swirl to mix.

After 48 hours, harvest cells to test for effectiveness of RNAi by RT-PCR or Western blot.

Comments:
For each gene, 4-6 target sites should be tested.
A nonspecific sequence can be used as a negative control.

Note for HSF1
I have used the target sequence aaatcaagaggaaagtgacc for HSF1 in MCF7 cells. Western analysis showed a 69 fold reduction in HSF1 protein. I still have to test for lack of HSF1 in a functional assay. So far in my hands, HeLa cells do not survive HSF1 RNAi.

References:
Silencer siRNA Construction Kit protocol (pdf file available online from Ambion)
siRNA user's guide: www.mpibpc.gwdg.de/abteilungen/100/105/

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