Protocol for vector-based expression of siRNA in ES cells

(Heiko Lickert)

This protocol is based on following publications:

Brummelkamp et al., 2002: Science 296: 550-553 (siRNA expression vector, pSUPER)
Thomas Tuschl, 2002: Nat. Biotech. 20: 446-447 (short review)
Hasuwa et al., 2002: FEBS Letters 532: 227-230 (siRNA in transgenic animals)
Kunath et al., 2003: Nat. Biotech. 21: 559-561 (shRNA transgenics using ES-tetraploid embryo/ aggregation)

The siRNA vector used was constructed by Dr. Jerry Gish, Pawson lab and is based on pcDNA3.1 (+). It contains the human RNAse P RNA H1 promoter. This promoter is transcribed by RNA polymerase III, which produces a small RNA transcript lacking a polyA tail, initiates transcription preferentially at +1 nucleotide adenosine or guanosine, and terminates transcription after five thymidines in a row. The cleaveage of the transcript at the termination signal is after the second uridine yielding the ends of synthetic siRNAs, which also contain two 3' overhanging T or U nucleotides.

<u>RNAi vector – series 2 (Figure 1)</u>

The human H1 promoter (100bp) was synthesized and cloned into pcDNA3.1 (+) (Invitrogen) thereby replacing the CMV promoter. The CMV promoter was released by BglII-XbaI digest (Kunath et al., 2003). The pcDNA3.1 vector contains Amp resistance and Neo selection marker. Two annealed oligonucleotides containing the sequence for the small hairpin RNA can be ligated into the 5'-Asp718 site and the 3'-XbaI site of the vector. The first nucleotide after the Asp718 restriction site is +1 position and should be either A or G. To sequence use pcDNA3.1/BGH reverse primer (5'-TAG AAG GCA CAG TCG AGG-3'). We use DMSO in the sequencing reactions since the target sequence forms secondary structure (ABI 377/ DGTP big dye/ and start of sequence as early as possible).
The vector can be linearized with ScaI (cuts in Amp) for electroporation into ES cells, which leaves enough buffer sequences on the 5'- and 3' borders. For further details see vector map (Figure1).

<u>Design of targeting sequence for RNAi (see cited publications and Figure 2)</u>

1. The target sequence should be in the coding region of the gene of interest and around 100 bp downstream of the Start-ATG and 100 bp upstream of the Stop-codon. The start and stop regions are most likely to be associated with regulatory proteins which may limit accessibility to the RISC complex.
2. For the design of the oligos start with sense target sequence followed by the 9bp loop and then the antisense target sequence followed by the Stop signal.
3. We use 19-23 bp target sequences with a GC content = 9 (+/- 1). The sequence should start with two adenosines followed by 19 nucleotides and ending with the

9bp stem loop (TT'CAA'GAG'A). If no sequence in the gene of interest matches these criteria, it is possible to start with AG, GA, or GG.

4. The stem loop is followed by the antisense target sequence and 5 thymidines (stop signal). In case the start is not AA we add another 2 thymidines in the end to make sure the RNA Pol III terminates proper.
5. Important: The target sequence should never contain more than 3 thymidines and adenines in a row, because of early termination!
6. It is also important that the target sequence doesn't form additional secondary structure to the stem loop. Check the 19-23 bp for equal distribution of Pu/Py and avoid palindromic sequences.
7. Blast the sequence against the database to avoid targeting of homologous sequences. Think about controls, target more than one region of your target gene to rule out off-target regulation. If you target a mouse sequence it might be possible to use a human cDNA (mismatsches) to rescue the knockdown.
8. Further papers: I. Interferon-response: Sledz et al., 2003; Nature Cell Biol. 9, 834-839. Bridge et al., 2003; Nat. Genet. 34, 263-64. Off-target regulation: Jackson et al., 2003; Nat. Biotech. 6, 635-; Chi et al., 2003, PNAS. Reviews: Hannon, 2002; Nature; Sharp, 2002; Nat. Rev. Genet.)
9. The QIAGEN page has a RNAi predictor, (http://python.penguindreams.net/Order_Entry/jsp/ShowOptions.jsp?Country=1&Proceed=Proceed). Hannon web page for siRNA design!!!

Electroporation into ES cells and functional assay for siRNA construct

Usually the pcDNA3.1-siRNA construct is linearized by Sca1 and 20-25 μg of DNA is electroporated into ES cells by standard procedures. Stable clones are picked after 7-10 days of G418 selection and expanded for freezing and testing. If the gene of interest is expressed in ES cells the efficiency of the RNAi can be tested by western, northern, or RT-PCR analysis. In case the gene is not expressed in ES cells it might be possible to differentiate ES cells to induce target gene expression. For alternatives see pSUPER paper.

In vivo analysis of RNAi targeted ES cells

We use siRNA-silenced ES cell aggregation to tetraploid embryos to produce completely ES cell-derived embryos, thereby allowing rapid analysis of the phenotype *in vivo* (Kunath et al., 2003). Either the ES cell or the tetraploid embryos should have an constitutive GFP marker to identify completely derived-ES cell embryos. The tetraploid aggregations are especially powerful if the gene of interest has an early embryonic lethal phenotype. For later phenotypes it is absolutely necessary to use hybrid ES cell lines, since ES-R1 derived embryos die shortly before birth. Hybrid ES cell lines give rise to viable offspring (see Rudi Jaenisch and Andras Naggy).

For analysis of non-embryonic lethal phenotypes it might be worth considering transgenic analysis (see Hasuwa et al., FEBS letters).