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Cloning of short hairpin RNAs for gene knockdown in mammalian cells

Patrick J Paddison[1], Michele Cleary[2], Jose Maria Silva[1], Kenneth Chang[1], Nihar Sheth[1], Ravi Sachidanandam[1] & Gregory J Hannon[1]

[1]Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, New York 11724, USA. [2]Rosetta Inpharmatics, 401 Terry Avenue N., Seattle, Washington 98109, USA.

RNA interference (RNAi) has become a methodology of choice for knocking down gene expression in a variety of biological systems[1,2]. The demonstration, in mammalian *in vitro* systems, of gene silencing using double-stranded RNA (dsRNA) products <30 base pairs (bp) in length has placed RNAi at the forefront of gene manipulation techniques in somatic cells[3–6]. Two types of dsRNA triggers are now commonly used to evoke RNAi in mammalian cells: (i) chemically or *in vitro*–synthesized small interfering RNAs (siRNAs)[3,4] and (ii) short hairpin RNAs (shRNAs) expressed from RNA polymerase III promoters[2]. We and others have chosen to explore the shRNAs for several reasons: first, the considerable cost of chemically synthesized siRNAs; second, the possibility of enforceable and stable expression of shRNAs; and third, the availability of applications of expression constructs in primary cell types (for example, using retroviruses) and in whole organisms (for example, in mouse). We have developed a system to drive expression of shRNAs by placing them under the control of the human RNA polymerase III U6 small nuclear RNA (snRNA) promoter, which normally controls expression of small RNAs in cells. This system has now been demonstrated to be effective both in *vitro*[2, 7–9] and *in vivo* transiently in mouse[10], stably during hematopoiesis[9] and stably in the generation of transgenic mice[11,12]. The pSHAG-MAGIC2 (pSM2) cloning vector (Fig. 1) is roughly equivalent to pSHAG-MAGIC1 (ref. 13) with a few notable exceptions. First, the new cloning strategy is based on the use of a single oligonucleotide that contains the hairpin and common 5′ and 3′ ends as a PCR template (Fig. 2). That is, the oligonucleotide itself serves as template and is amplified by PCR using universal primers that contain an *Xho*I site (within the 5 primer) and an *Eco*RI site (within the 3′ primer) to facilitate cloning (Fig. 3). The resulting PCR fragments are then cloned into the hairpin cloning site of the vector pSM2, the clones are verified by sequencing, and the construct is introduced into the appropriate cell lines, where expression of the miR-30–styled hairpins is driven by the human U6 promoter.

MATERIALS

REAGENTS

Amplification buffer (NEB; provided with Vent DNA polymerase) and Vent DNA polymerase (NEB)
GC-melt PCR reagent (Clontech; provided with Advantage-GC PCR kit)
Dimethyl sulfoxide (DMSO; Sigma)
dNTP solution (10 mM)
Primer oligonucleotides (the sequences shown in Fig. 3) and Template oligonucleotide (as described in step 1; example in **Fig. 2**)
Phenol/chloroform , 1:1 (vol/vol), and 10% chloroform
0.3 M sodium acetate (pH 4.8)
70% (vol/vol) ethanol
Glycogen carrier (Boehringer, Mannheim)
High-melting-temperature agarose (such as MetaPhor Agarose, Cambrex)
Glass bead kit (such as QiaexII gel extraction kit, Qiagen)
Restriction endonucleases *Eco*RI and *Xho*I
PirPlus (PIR1)-competent bacteria (Open Biosystems) and selective antibiotics (chloramphenicol and kanamycin)
T4 DNA ligase (NEB, high-concentration ligase)
Vector: pSM2 (pShag Magic Version 2.0; Open Biosystems)

EQUIPMENT

Thermal cycler programmed with the desired amplification protocol

Design of the template oligonucleotide

PROCEDURE

1| To generate the hairpin primer, select a 'sense' sequence(s) of 22 nucleotides (nt) in length from the coding sequence of the gene of interest for each clone to be constructed. *Only coding sequences are targeted, and each shRNA should be chosen such that it contains more than three mismatches to any other gene. Known single-nucleotide polymorphisms should be avoided, as should common exons targeted in alternatively spliced mRNAs. It is recommended that you create at least 3–6 distinct shRNA clones for each gene to be studied. The link to the hairpin generation program 'RNAi oligo retriever' can be found at www.cshl.org/public/SCIENCE/hannon.html. Either accession numbers from GenBank or raw sequences or siRNA sequences can be used to generate hairpin PCR primers.*

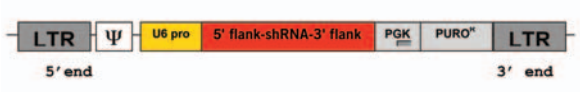

Figure 1 | Retroviral backbone of pSHAG-MAGIC2. The shRNA expression cassette is carried in a validated murine stem cell virus (MSCV) backbone. The 5′ and 3′ flanks are derived from 125 bases of sequence surrounding the human miR-30 miRNA. The pSM2 vector is a redesign of pSM1 that is more stable and less prone to recombination. This vector can be used both for transient delivery by transfection and for stable delivery using the replication-deficient retrovirus as a delivery method. The complete vector sequence is available at www.cshl.org/public/SCIENCE/hannon.html and www.openbiosystems.com.

2| Complete the design of the template oligonucleotide by incorporating miR-30 microRNA (miRNA) sequences (as DNA) into the target sequence (see **Fig. 2** for guidance). Insert the miR-30 loop sequence between the sense and the antisense sequences and add the appropriate flanking sequences of miR-30 miRNA to the 5′ end and the 3′ end of the target.
Figure 2 shows the configuration of a sample template DNA oligonucleotide derived from the RNA oligonucleotide design. **▲CRITICAL STEP**

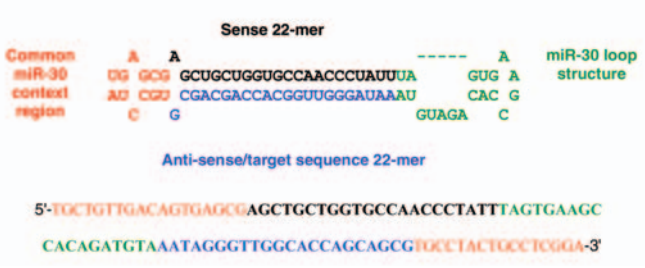

Figure 2 | Design and sequence of a sample template. Above, the final, preprocessed structure of the miR-30–styled shRNA, with derivations of each part of the sequence. Below, the corresponding oligonucleotide DNA template sequence design derived from the oligonucleotide RNA. The sequences in red represents the flanking miR-30 sequences and those in green the miR-30 loop structure. The sample sense- and antisense-selected target sequences are shown in black and in blue, respectively. The mir-30–styled shRNA is synthesized as a single-stranded DNA oligonucleotide with common ends corresponding to part of the endogenous miR-30 miRNA flanking sequence. The flanking regions, shown in red, are used as universal flanks to prime a reaction, whereby the entire miR-30–styled shRNA is amplified to produce a PCR product that may be cloned into pSM2.

3| Synthesize or order the template oligonucleotide designed in steps 1 and 2 and the 5′ and 3′ primer oligonucleotides (Fig. 3).
Because very little primer is required for the PCR reaction, these can be ordered at 0.5 μmol scale from, for example, Sigma-Genosys. We find purification of the oligonucleotides by polyacrylamide gel electrophoresis to be costly and unnecessary.

Amplification of the template oligonucleotide

4| Set up the amplification reaction (one for each short hairpin sequence to be tested).

For each 100-μl reaction:			
10× amplification buffer	10 μl	3′ primer (50 μM stock)	1 μl
DMSO	5 μl	Template oligonucleotide (100 ng/μl)	1 μl
GC melt PCR reagent	5 μl	Vent DNA polymerase	1 μl
dNTPs (10 mM stock)	2 μl	Water	74 μl
5′ primer (50 μM stock)	1 μl		

Note that the GC-melt PCR reagent is available from Clontech only in the Advantage-GC PCR kit, not as an individual reagent. This PCR kit, however, can be used for individual miR-30 shRNA cloning experiments with reasonable success.

5| Amplify the nucleic acids according to the following program:

Cycle number	Denaturation	Annealing	Polymerization	Final
1	1 min at 94 °C	30 s at 54 °C	1 min at 75 °C	
2–24	30 s at 94 °C	30 s at 54 °C	1 min at 75 °C	
Last	30 s at 94 °C	30 s at 54 °C	11 min at 75 °C	
Hold				4 °C thereafter

Times and temperatures may need to be adapted to suit the particular reaction conditions.
➨TROUBLESHOOTING

6| Analyze an aliquot of the amplification reaction by electrophoresis through a polyacrylamide gel, and estimate the concentration and yield of the amplified target. The reaction should produce a single band of 137 bp.

7| To purify the amplification product, extract the reaction once with an equal volume of phenol/chloroform and once with an equal volume of chloroform.
If using a column-based PCR cleanup kit, make sure that the procedure is designed to capture products as small as 100 bp.

5'miR30PCRxhoIF (5' primer):
5'-CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCG-3'

3'miR30PCREcoRIF (3' primer):
5'-CTAAAGTAGCCCCTTGAATTCCGAGGCAGTAGGCA-3'

Figure 3 | Primer oligonucleotides for amplification. The universal PCR primers shown here contain *Xho*I and *Eco*RI cloning sites that allow cloning into *Xho*I and *Eco*RI sites in the pSM2 miR-30 cloning cassette. The portions of sequences in red represent the regions that are complementary to the target shown in **Figure 2**.

8| Recover the DNA by precipitation with ethanol in the presence of 0.3 M sodium acetate (pH 4.8) and wash the pellet in 70% ethanol.
We recommend adding glycogen (at 50 μg/ml final concentration) carrier as a pellet marker.

Cloning of the amplification product into pSM2

9| Dissolve the pellet in 10 mM Tris (pH 7.6) or water and digest the amplification product with *Eco*RI and *Xho*I at 15–25 °C for 90 min.

10| Purify the resulting fragment by electrophoresis through 2% high-melting-temperature agarose, recover the DNA from the gel using the QiaexII glass bead gel purification kit, and resuspend the DNA in 20 μl of 10 mM Tris (pH 7.8). The resulting fragment will be ~114 bp.
Metaphor agarose gives excellent resolution for these separations but is not essential.

11| Prepare the vector DNA by digesting 2 μg of pSM2 with *Eco*RI and *Xho*I, gel purify the linearized plasmid and resuspend it in 20 μl of 10 mM Tris (pH 7.8).

12| Set up the ligation reaction, including 3–5 μl of PCR product, 1 μl of the vector, and T4 DNA ligase, in a 20-μl reaction. Incubate the reaction at 15–25 °C for 10 min to 2 h, as appropriate.

13| Transform 5–10 μl of the ligation reaction directly into PIR1-competent bacteria and select for growth in the presence of both chloramphenicol and kanamycin (each at a concentration of 25 μg/ml).
➨TROUBLESHOOTING
▲CRITICAL STEP

Sequencing and transformation of the cloned shRNA

14| Select and verify by sequencing at least six Cm^R/Km^R bacterial clones for each individual target oligonucleotide when cloning individual shRNAs, or ensure at least threefold coverage when sequencing complex cloning pools (that is, 3–10,000 shRNAs).
The sequence of the U6 sequencing primer (beginning at –42 from the start of transcription) is 5′-GTAACTTGAAAGTATTTCG-3′.

As a matter of practice, we only use sequence-verified shRNAs for biological experiments. We find that the quality of oligonucleotide synthesis can vary considerably with regard to errors introduced during synthesis.

➨TROUBLESHOOTING

15| Transform the verified clones into the cell lines of interest to test their ability to knock down gene expression. The vector(s) may be transiently transfected into any commonly used cell line that also expresses the gene of interest, or transiently transfected with the target gene into a cell line that does not express the gene of interest.

16| Monitor expression of the target gene in the transfected cells by western or northern blotting or by reverse transcriptase-PCR (RT-PCR).

▲CRITICAL STEP

17| Identify the clones that produce the desired knockdown effect and proceed with stable expression experiments to further analyze loss of gene expression.

▲CRITICAL STEP

TROUBLESHOOTING TABLE

PROBLEM	SOLUTION
Steps 5 and 14 The sequences of selected clones do not always match the sequence of the template used in amplification and cloning.	The biggest problem that we have encountered with this protocol is the error frequency associated with PCR amplification. As it turns out, the oligonucleotides used as PCR templates are predicted to fold back on themselves as single-stranded DNA hairpins and have relatively high melting temperatures, often >75 °C. Therefore, we do not recommend deviating from the protocol described above. We have now successfully cloned and sequenced over 45,000 miR-30–styled hairpins using this specific method.
Step 13 We sometimes obtain a high background upon transformation of the ligation reaction products into PIR1-competent cells.	The pSHAG-MAGIC vectors were designed with two bacterial drug selection markers, one on the backbone of the vector (Km^R) and one situated next to the hairpin expression cassette (Cm^R). To minimize background and ensure a successful short hairpin activated gene silencing (SHAG), it is paramount that both markers be used in transformation of PirPlus (PIR1) cells.

▲CRITICAL STEPS

Step 2 The flanking regions of the template (**Fig. 2**; shown in red) are used as universal flanks for PCR amplification. These 5′ and 3′ ~125-nt flanks of the endogenous human miR-30 miRNA were modified to contain *Xho*I and *Eco*RI sites, respectively (see www.open.biosystems for map and sequence). The universal PCR primers (**Fig. 3**) contain *Xho*I and *Eco*RI cloning sites that allow cloning into *Xho*I and *Eco*RI sites in the pSM2 miR-30 cloning cassette. The modified miR-30 context (**Fig. 2**) allows for efficient processing of both the wild-type miR-30 miRNA sequence as well as miR-30–styled shRNAs (data not shown; see also ref. 14).

Step 13 Ligation mixtures must be transformed into PIR1-competent bacteria. The pSM2 plasmid harbors a conditional bacterial origin of replication that requires expression of the *pir1* gene to be rendered functional.

Step 16 For small-scale applications using shRNAs (for example, knocking out a handful of genes), we strongly recommend constructing 3–6 shRNAs per gene and carrying out a validation step before doing the actual biological experiment. For example, we often will transiently transfect shRNA plasmids into any commonly used cell line that also expresses the gene of interest and assay efficacy of the shRNA by western blotting, northern blotting or RT-PCR. Alternately, the target gene can be introduced transiently along with the shRNA and assayed in a cell type lacking endogenous expression. Western blots or RT-PCR are good predictors of efficacy in these assays. We find a direct correlation between whether an shRNA works well in transient assays and whether it works well when expressed stably (for example, from a

retrovirus). In fact, we have been able to build an 'epiallelic' series by this method: a series of shRNAs with different efficacies that give rise to phenotypes of corresponding severity. The correlation of knockdown and phenotype appears to hold true from transient and stable experiments *in vitro* to stable expression *in vivo*[9].

Step 17 One particular plasmid design will not suit all needs. The pSM2 vector therefore is constructed so as to permit high-throughput, automated transfer of the inserted shRNAs between different vector backbones. This feature will permit, for example, rapid migration of the library into other delivery systems, such as lentiviruses and adenoviruses. It will also permit the design of customized delivery vehicles suitable to each screening application.

COMMENTS

An interesting difference with RNAi in mammals is the apparent lack of amplification and transport of the silencing trigger. In contrast to what occurs in *Caenorhabditis elegans* or in plants, mammalian RNAi appears to produce a transient, cell-autonomous targeting effect. These triggers of RNAi in mammalian cells have half-lives and, at least for the time being, must be fed continuously to the RNAi machinery to maintain silencing. Because stable RNAi requires stable expression of the dsRNA trigger, we and others have developed retroviral expression constructs that promote the stable expression of shRNAs[2,9,12,15]. In the particular retroviral vector described in this protocol (pSM2), the shRNA cassette is placed between the 5′ long terminal repeat (LTR) and the drug-selection markers (**Fig. 1**), and the design of the hairpin cassette incorporates sequences of the human miR-30 miRNA (**Fig. 2**). First, adding the miR-30 loop and 125 nt of miR-30 flanking sequence on either side of the hairpin results in more than tenfold greater Drosha and Dicer processing of the expressed hairpins, as compared to the old designs (P.J.P., D. Siolas, A.M. Denli and G.J.H., unpublished observations; see refs. 14,16,17). Increased Drosha and Dicer processing translates into greater siRNA production and greater potency for expressed hairpins. Second, by using the miRNA-30 designs, we can incorporate 'rule-based' designs for target sequence selection. One such rule is the destabilization of the 5′ end of the antisense strand, which results in strand-specific incorporation of miRNAs into RISC[18]. Last, the miR-30 design offers more flexibility in applications of gene silencing, as these sequences can also be expressed from RNA polymerase II promoters (for example, cytomegalovirus) or even arrays of different hairpins in polycistronic transcripts (data not shown; see ref. 19).

SOURCE

G.J. Hannon is the editor of *RNAi: A Guide to Gene Silencing* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, 2003). This protocol is a new development of ideas discussed in that volume.

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