

GENERATION OF HAIRPIN-BASED RNAI VECTORS FOR BIOLOGICAL AND THERAPEUTIC APPLICATION

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Abstract

RNA interference (RNAi) is a natural process of gene silencing mediated by small RNAs. Shortly after the discovery of the RNAi mechanism, scientists devised various methods of delivering small interfering RNAs (siRNAs) capable of co-opting the endogenous RNAi machinery and suppressing target gene expression based on sequence complementarity. RNAi has since become a powerful tool to study gene function and is being investigated as a potential

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therapeutic approach to treat a vast array of human diseases (e.g., cancer, viral infections, and dominant genetic disorders). Among the available RNAi vectors are hairpin-based expression platforms (short-hairpin RNAs and artificial microRNAs) designed to mimic endogenously expressed inhibitory RNAs. These RNAi vectors are capable of achieving long-term potent gene silencing *in vitro* and *in vivo*. Here, we describe methods to design and generate these hairpin-based vectors and briefly review considerations for downstream applications.

1. INTRODUCTION

RNA interference (RNAi) is an evolutionarily conserved cellular process involved in gene regulation and innate defense (Krol *et al.*, 2010). RNAi directs sequence-specific gene silencing by double-stranded RNA (dsRNA) which is processed into functional small inhibitory RNAs (~21 nt) (Provost *et al.*, 2002). In nature, small RNAs known as microRNAs (miRNAs) are the key mediators of this gene regulatory process (Krol *et al.*, 2010). Mature miRNAs (~19–25 nts) are excised from stem-loop regions within larger primary miRNA transcripts (pri-miRNAs). A cascade of cleavage reactions catalyzed by the ribonucleases, Drosha-DGCR8 and Dicer (Gregory *et al.*, 2004; Han *et al.*, 2004; Lee *et al.*, 2003), releases the miRNA duplex. A single strand (the antisense “guide” strand) subsequently enters the RNA-induced silencing complex (RISC) (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003), thus producing a functional complex capable of base pairing with target transcripts and reducing their expression by various mechanisms. Perfect or near perfect binding along the length of the small RNA induces target transcript cleavage, whereas imperfect complementarity (typically to transcript 3'-UTRs) causes the canonical miRNA-based repression mechanism resulting in translational repression and mRNA destabilization (Guo *et al.*, 2010; Lewis *et al.*, 2005).

RNAi was initially discovered as a perplexing gene silencing observation in plants and worms (Ecker and Davis, 1986; Fire *et al.*, 1998; Napoli *et al.*, 1990). Over the past decade, our increased understanding of miRNA biogenesis and gene silencing mechanisms has facilitated the development of various strategies for co-opting the cellular RNAi machinery to direct specific silencing of virtually any gene. These RNAi-based technologies have become invaluable molecular tools to study gene function and are being investigated as therapeutic reagents for many human diseases. The potential to artificially induce gene silencing depends on our ability to design inhibitory RNAs that properly engage the RNAi machinery and to introduce them into target cells or tissues. The central RNAi effectors, known as small interfering RNAs (siRNAs), are designed to mimic mature miRNA duplexes, but with the guide strand exhibiting

perfect complementarity to the intended target transcript to trigger the potent cleavage-based silencing mechanism. siRNAs are typically synthesized *in vitro* using modified bases for improved stability, specificity, and reduced immunostimulatory properties (Behlke, 2008). Upon entering the cell by endosomal uptake and escape or by electroporation, siRNAs engage the RNAi pathway at the Dicer-to-RISC stage. Alternatively, siRNAs can be incorporated into expression-based systems by embedding the sequences into stem-loop structures designed to mimic pre-miRNAs (short-hairpin RNAs or shRNAs) or pri-miRNAs (artificial miRNAs) (Paul *et al.*, 2002; Zeng *et al.*, 2002). shRNAs are generally expressed from vector systems, most often with strong, constitutive Pol III promoters (e.g., U6 and H1) (Paul *et al.*, 2002; Sui *et al.*, 2002). In order to mimic pre-miRNAs, shRNAs are transcribed as sense and antisense sequences connected by a loop of unpaired nucleotides. Following transcription, shRNAs are exported from the nucleus by Exportin-5 and, once in the cytoplasm, are cleaved by Dicer to generate functional siRNAs (Lund *et al.*, 2004; Provost *et al.*, 2002; Yi *et al.*, 2003). More recently, scientists have embedded siRNA sequences into molecular scaffolds designed to mimic pri-miRNAs which enter the RNAi pathway upstream of Drosha-DGCR8 (Chung *et al.*, 2006; Zeng *et al.*, 2002). These artificial miRNAs more naturally resemble endogenous RNAi substrates, thus improving the efficacy and accuracy of downstream processing events (Boden *et al.*, 2004; Silva *et al.*, 2005). Furthermore, since miRNA hairpins can be embedded within larger transcripts, this approach is more amenable to Pol II-based expression systems, providing enhanced potential for tissue-specific and inducible gene silencing. Expression-based RNAi vectors afford unique opportunities for employment of viral-based delivery systems, stable long-term gene suppression, and finer control of spatiotemporal silencing, among other related advantages associated with transgenic approaches (McJunkin *et al.*, 2011; Shin *et al.*, 2006).

With an array of RNAi triggers and delivery modalities available, researchers must decide which combination will yield a suitable inhibitory RNA dose to achieve potent and highly specific silencing in their experimental setting. Dose optimization remains a crucial consideration given the potential for RNAi treatments to induce cellular toxicity. High levels of exogenously supplied RNAi substrates may disrupt cellular function by saturating the RNAi machinery, thus interfering with natural miRNA-mediated gene regulation (Boudreau *et al.*, 2009a; Castanotto *et al.*, 2007; Grimm *et al.*, 2006). Also, artificial inhibitory RNAs have the potential to bind to and regulate unintended mRNA targets, an effect known as off-target gene silencing (Chi *et al.*, 2003; Jackson *et al.*, 2003; Semizarov *et al.*, 2003). Off-targeting primarily occurs when the seed region (nucleotides 2–8 of the small RNA) pairs with 3'-UTR sequences of unintended mRNAs and directs translational repression and destabilization of those transcripts, similar to the canonical miRNA-based silencing mechanism (Birmingham *et al.*, 2006;

Jackson *et al.*, 2006). Together, these adverse events may have severe consequences; for example, Grimm *et al.* reported that high-level shRNA expression, from strong Pol III promoters, in mouse liver induced fatality (Grimm *et al.*, 2006). Additional work from our laboratory identified that artificial miRNAs may have lower toxicity potentials. In comparison studies, we found that shRNAs are more potent but induce toxicity *in vitro* and *in vivo*, whereas artificial miRNAs are expressed at tolerably lower levels yet maintain potent gene silencing capacities (Boudreau *et al.*, 2008, 2009a; McBride *et al.*, 2008). Together, these results and others underscore the need to consider and monitor dosing in RNAi experiments. Controlling the dose of synthetic siRNAs is rather uncomplicated. By contrast, inhibitory RNA levels produced by expression-based systems are influenced by many factors (e.g., vector platforms, delivery modalities, promoter selection, hairpin structure, and availability of RNAi pathway components) which are likely to be unique to each experimental setting. Researchers should consider these factors in choosing an appropriate RNAi approach for their purposes.

Here, we provide methods to generate and screen hairpin-based RNAi vectors and discuss the downstream utility and considerations for these vectors in biological and therapeutic applications.



2. SELECTING CANDIDATE siRNA SEQUENCES

2.1. Retrieve sequences for your desired target transcript(s)

Choose your target gene(s) of interest and retrieve the relevant mRNA sequence(s) from NCBI, Ensembl, UCSC Genome Browser, or other available databases. We leave it to the reader's responsibility to further investigate target transcripts for variants which may result from RNA processing events (e.g., alternative splicing, alternative polyadenylation, and RNA editing, among others) that may influence the ability to target certain regions within the transcript. In general, we target the coding region; however, targeting the 5'- and 3'-UTR sequences is possible. Careful consideration for the target sequence with regard to the project objectives is important before proceeding with design and screening of inhibitory RNA sequences. For instance, the reader should consider whether allele- or splice-isoform-specific silencing is desirable or whether sequence conservation is important, allowing the RNAi vectors to be tested in multiple species.

2.2. Select 22-nt long target sites

Identifying potent and highly specific siRNA sequences is not trivial. Numerous empirical evaluations of large-scale siRNA knockdown data have allowed researchers to establish several siRNA design guidelines

(Khvorova *et al.*, 2003; Matveeva *et al.*, 2007). For example, one key consideration is that siRNA sequences be selected or manipulated to promote accurate loading of the antisense guide strand into RISC, leaving the sense strand to be degraded (Leuschner *et al.*, 2006; Matranga *et al.*, 2005). Furthermore, GC-content and positional nucleotide preferences also influence siRNA efficacy. Given the multifaceted nature of designing optimal siRNAs, we direct the reader to additional literature on the subject (Birmingham *et al.*, 2007; Davidson and McCray, 2011; Jackson and Linsley, 2010). Also, there are numerous publicly available siRNA design tools online. It is important to note that siRNA design rules serve more as guidelines, and that sequences adhering to them may not silence and vice versa. To date, no algorithm guarantees silencing efficacy, and most recommend the user pick three to four candidates for screening. Here, we will describe a basic strategy for siRNA target site selection which incorporates the most important criteria for promoting efficacy, in addition to certain rules that are specific to the design of hairpin-based RNAi expression vectors.

We identify 22-nt target sites within the target transcript that adhere to four criteria: (1) high propensity to primarily load the antisense guide strand into RISC, (2) GC content between 20% and 70%, (3) void of restriction enzyme sites relevant to downstream applications (e.g., cloning RNAi expression cassettes into viral vector systems), (4) lacking a stretch of four continuous A or T nucleotides (i.e., AAAA or TTTT). The latter prevents premature transcription termination from Pol III promoters which typically terminate at stretches of four to six T's. Strand biasing is determined by the thermodynamic stabilities present at the ends of the siRNA duplex. To achieve faithful loading of the antisense strand, the duplex must be designed such that there is strong G–C base pairing present at the 5'-end of the sense (passenger) strand and weak A/G–U base paring at the opposing terminus (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003). The RISC complex selects the strand with the weakest 5'-end thermodynamic stability, in this case, the antisense strand (Fig. 14.1). Hence, we select target sequences that have G or C nucleotides at positions 3 and 4 (*note*: positions 1 and 2 correspond to the dinucleotide 3'-overhang of the antisense strand) and A, T, or C nucleotides at positions 20, 21, and 22. The C is allowed at the latter positions because we can destabilize the resulting G–C base pairs by converting them to G–U base pairs (G and U weakly pair in RNA) without altering the antisense strand sequence. Next, the GC content of the 22-nt site is calculated by dividing the number of G or C nucleotides by 22. For instance, the target site shown in Fig. 14.1 has 12 G or C nucleotides, resulting in a GC content of 55% which is within the acceptable range. Finally, we avoid stretches of A's and T's and relevant restriction enzyme sites for reasons mentioned above. *Note*: the user must also avoid creating these elements upon incorporating the siRNA into shRNA or artificial miRNA scaffolds.

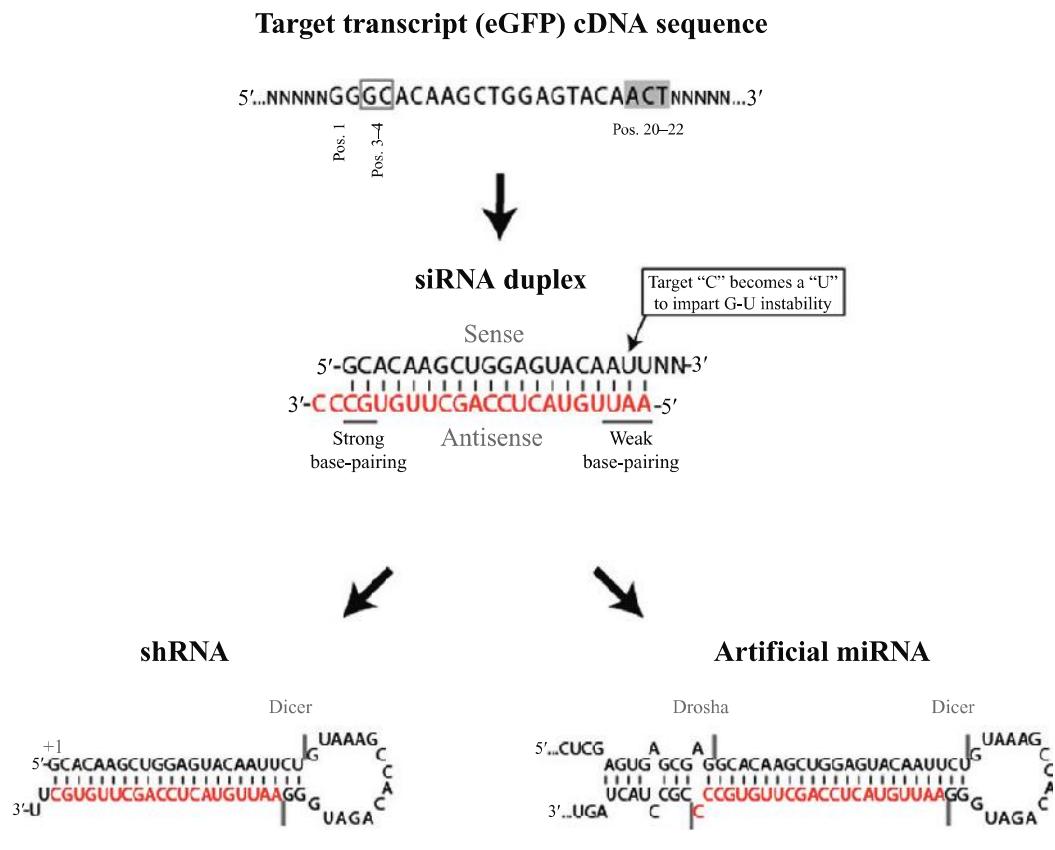


Figure 14.1 siRNA sequence selection for hairpin-based vectors. Schematic depicting the design of an eGFP-targeted siRNA sequence which satisfies the rules outlined in Section 2.2. siRNA sequences may be embedded into shRNA or artificial miRNA scaffolds for expression.

3. CLONING shRNA EXPRESSION CASSETTES

3.1. Notes on siRNA sequence selection for shRNA application

The criteria defined above are applicable with the following considerations. siRNAs designed for embedding into a shRNAs transcribed from the Pol III U6 promoter should contain a G nucleotide at position 3 to allow for proper expression and strand biasing of the resulting siRNA. U6-driven transcripts initiate at the +1-G position located at the 3'-end of the U6 promoter. By contrast, the H1 promoter (another Pol III promoter) can initiate with any nucleotide at the +1 position. Pol III-based transcription terminates at a stretch of four to six T's, resulting in two to three U's being incorporated at the 3'-end of the shRNA (Fig. 14.1). This provides the short 3'-overhang on the hairpin which is optimal for downstream processing events (e.g., nuclear export and subsequent recognition and cleavage by

Dicer) (Vermeulen *et al.*, 2005; Zeng and Cullen, 2004). Thus, the presence of A or G nucleotides, which are both capable of base pairing with U, at positions 1 and 2 of the target sequence may promote better silencing efficacy. However, there is considerable empirical evidence supporting that shRNAs may be effective silencers even without base pairing at these terminal positions.

3.2. Oligo design and cloning protocol for shRNA-tailed PCR

The shRNA cassette will be designed for transcription by the mouse U6 promoter as sense, loop, and antisense sequences followed by the Pol III terminator. This results in a hairpin transcript that has a short 3'-overhang necessary for downstream processing by the RNAi pathway machinery (Fig. 14.1, shRNA). Positioning the antisense region at the 5'-end (i.e., antisense–loop–sense) is also possible but not described here. Steps 1–6 are outlined in Fig. 14.2 and describe the incorporation of a siRNA (targeting eGFP as the example) into a shRNA expression vector.

Step 1. Layout the shRNA DNA template.

The shRNA DNA template consists of the following from 5' to 3': (1) the sense sequence consisting of nucleotides 3–22 of the target site, (2) the 19-nt loop sequence (5'-CTGTAAAGCCACAGATGGG-3') which is partially derived from the naturally occurring human miR-30 transcript, and (3) the antisense sequence which is the reverse complement of the target site (positions 3–22).

Step 2. Convert C's to T's at the sense 3'-end to impart duplex instability.

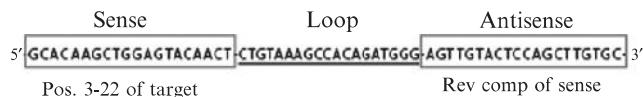
As mentioned above, proper loading of the antisense strand requires weak base pairing at its 5'-end in the siRNA duplex and strong base pairing opposite. Target sequence selection allows C's to be in 3'-end of the sense strand (positions 19–22 of the target site) because the C's, which will be paired with G's on the antisense strand, can be converted to U's, which pair much more weakly to G's. Thus, only the sense strand is manipulated to promote loading of the antisense strand. In the example, the terminal three nucleotides of sense sequence is converted from ACT to ATT.

Note: at this point, it is good practice to ensure that the hairpin sequence is void of stretches of four or more T's and any restriction enzyme sites (e.g., *Xba*I and *Eco*RI) relevant to downstream applications.

Step 3. Convert all T's to U's and fold RNA using UNAfold.

Convert the shRNA DNA template into an RNA transcript by replacing the T nucleotides with U's and use the UNAfold RNA folding tool (available at <http://www.idtdna.com/Scitools/Applications/unafold/>) to confirm that the shRNA transcript

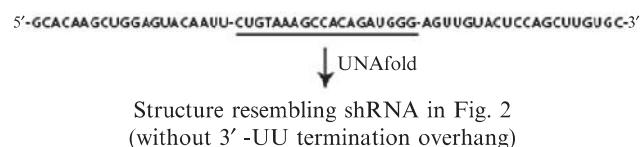
Step 1. Layout the shRNA DNA template.



Step 2. Convert C's to T's at sense 3'-end to impart duplex instability.



Step 3. Convert all T's to U's and fold RNA using UNAFold.



Step 4. Add U6 promoter, terminator, and XhoI sequences to shRNA DNA template.



Step 5. Reverse complement the sequence and order DNA oligos (forward and reverse).

U6-shRNA reverse primer



U6 forward primer



Step 6. PCR amplify U6-shRNA expression cassette, TOPO clone, and screen.

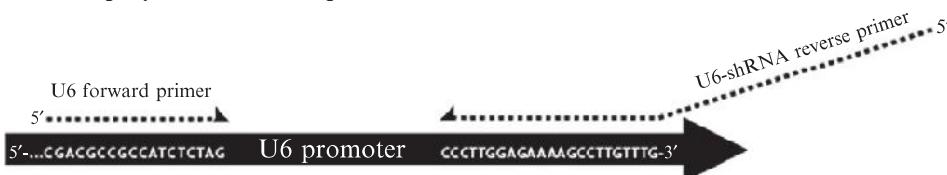


Figure 14.2 shRNA cloning scheme. The details for designing shRNAs and performing tailed PCR to generate the mU6-driven shRNA expression cassette are described in the text (Section 3.2, Steps 1–6). Shown here is the example of incorporating the eGFP-targeted siRNA into the shRNA expression platform.

forms the desired secondary structure. Note: select “RNA” as the input “nucleotide type” at the user interface and leave the remaining parameters set to default. UNAfold generally predicts several possible secondary structures for each input; however, the correct version of the shRNA should be at the top as the most stable structure. In this case, the fold should resemble the shRNA depicted in Fig. 14.1, but lacking the 3'-UU overhang resulting from Pol III termination.

Step 4. Add U6 promoter, terminator, and *Xho*I sequences to the shRNA DNA template.

To generate the shRNA expression cassette, a tailed PCR strategy will be used to amplify the mouse U6 promoter sequence while tailing on the necessary shRNA components and a restriction enzyme site for identifying full-length PCR products. The 3'-end of the U6 promoter sequence (5'-CCCTTGGAGAAAAGCCTTGTTC-3'; minus its 3'G) is placed upstream of the shRNA sequence. This will serve as the priming site during the initial PCR cycles. Next, place the Pol III terminator sequence (six T's) followed by an *Xho*I restriction enzyme site (5'-CTCGAG-3') downstream of the shRNA sequence.

Step 5. Reverse complement the sequence and order DNA oligos (forward and reverse).

To easily reverse complement DNA sequences, there are several tools available online (e.g., http://www.bioinformatics.org/sms/rev_comp.html). We typically add four A nucleotides to the 5'-end of the reverse-complemented oligo before ordering. The resulting shRNA-tailed reverse oligos can be ordered at reasonable cost from a variety of commercial vendors (e.g., Integrated DNA Technologies or Sigma-Aldrich) using the smallest synthesis scale available and standard desalting purification. A forward primer corresponding to the 5'-end of the mouse U6 promoter (5'-CGACGCCGCCATCTCTAG-3') should also be ordered. Upon receipt, the oligos can be reconstituted in purified water. In general, we make a 100 μ M stock for storage and a 20- μ M working stock for use in PCR.

Step 6. PCR amplify the U6-shRNA expression cassette, TOPO clone and screen.

Step 6.1. Prepare and run the PCR as follows:

Reaction conditions

5 μ l 10 \times reaction buffer with MgCl₂
1 μ l mU6 forward primer (20 μ M)
1 μ l shRNA-tailed reverse primer (20 μ M)
1 μ l dNTPs (10 mM)
1 μ l of mU6 containing plasmid (Tb:mU6; 10 ng/ μ l)
0.5 μ l enzyme mix (i.e., polymerase)
40.5 μ l dH₂O

Amplify in a thermocycler

94°C for 3 min (initial denaturation)
30 cycles of 94°C for 30s, 54°C for 30s, and 72°C
for 30s
72°C for 5 min (final extension)

- Step 6.2.** Confirm amplification of the desired PCR product size. Run 5 μ l of the PCR on a 1% agarose gel to verify the presence of a single band (\sim 400bp) in size.
- Step 6.3.** Perform TOPO cloning reaction as per the manufacturer's protocol by mixing 4 μ l of the PCR product with 1 μ l of salt solution and 1 μ l of pCR[®]4-TOPOTM cloning vector, and incubating the reaction for 10 min at room temperature.
- Step 6.4.** Transform 2–3 μ l of the TOPO cloning reaction into chemically competent *Escherichia coli* using standard procedures and plate onto ampicillin-selective plates for incubation overnight at 37°C. Note: This selects against the undesired template Tb:mU6 plasmid which is kanamycin resistant, allowing for positive selection of the desired pCR[®]4-TOPOTM plasmids which confer ampicillin resistance and kanamycin resistance.
- Step 6.5.** Pick 6–12 colonies and inoculate each into 3ml of LB growth medium containing 50 μ g/ml ampicillin. Grow overnight in a shaker at 37°C.
- Step 6.6.** Isolate plasmid DNA using standard procedures or a commercial miniprep kit. We recommend using the Qiaprep Miniprep kit (Qiagen) which yields plasmid DNA of sufficient quality for cell culture applications.
- Step 6.7.** Screen for positive full-length shRNA clones by digesting the plasmids with *Xba*I and performing gel electrophoresis. The presence of a band at \sim 400bp indicates that the U6-shRNA cassette is in reverse orientation, while a \sim 50-bp band indicates forward insertion.
- Step 6.8.** Sequence positive clones using an automated sequencer that employs Sanger-based fluorescent chemistry. The cycling parameters may need to be optimized to sequence through the hairpin structure. For example, we previously found that adding DMSO (to 4%, v/v) to the sequencing reaction and using a longer initial denaturation step produced more reliable and consistent sequencing through the shRNAs. M13 forward or reverse primers can be used for sequencing TOPO cloning insertions.

The PCR for generating the shRNA expression cassettes is similar to a standard PCR which requires a DNA template (in this case, a plasmid containing the mouse U6 promoter; TOPOBluntII:mU6 (Tb:mU6)), forward and reverse primers, dNTPs, reaction buffers, and DNA polymerase. Here, we use the Expand High-Fidelity PCR System (Taq-based, available from Roche); however, in our experience, shRNA-tailed PCRs have also proven successful with a

variety of proof-reading polymerases (e.g., Accuprime Pfx, Invitrogen or Phusion, New England Biolabs). With these enzymes, the blunt-ended PCR products can be sub-cloned into pCR®-Blunt-II TOPO, requiring removal of the kanamycin-resistant template plasmid (e.g., by DpnI digestion) prior to TOPO cloning.

3.3. Materials for shRNA cloning

Tb:mU6 plasmid (PCR template)
DNA oligos (mU6 forward and shRNA-tailed reverse primers)
Expand High Fidelity Polymerase and buffers (Roche)
Restriction enzymes and buffers (*Xho*I)
Gel electrophoresis equipment and reagents
pCR®4-TOPO TA cloning kit (Invitrogen)
Chemical competent bacterial cells
Ampicillin-containing LB agar growth plates
LB growth media
Qiaprep Miniprep kit (Qiagen)



4. CLONING ARTIFICIAL miRNA EXPRESSION VECTORS

4.1. Considerations for designing artificial miRNAs

Artificial miRNAs, or miRNA shuttles, are designed to mimic naturally occurring pri-miRNAs, for which the Drosha-DGCR8 and Dicer cleavage sites have been mapped and experimentally validated. With this information, the identity of the small RNA duplex which is processed from the initial stem-loop transcript is known. For artificial miRNAs, this region is replaced by siRNA duplexes, thus creating a miRNA-based hairpin which serves to shuttle siRNA sequences into the RNAi pathway. An important consideration for designing artificial miRNAs is to maintain the structural and sequence recognition motifs required for appropriate processing. Drosha-DGCR8 binds to regions of single-stranded nature located at the base of the pri-miRNA stem loop (Han *et al.*, 2004; Zeng and Cullen, 2005). Thus, including 50–100nts of the flanking sequences (5' and 3') native to the pri-miRNA will help to ensure that the stem-loop base folds properly to promote cleavage at the intended site. Artificial miRNAs have been generated using a number of naturally occurring pri-miRNAs as scaffolds for siRNA sequences (Chung *et al.*, 2006; Tsou *et al.*, 2011; Zeng *et al.*, 2002). Here, we will describe a method to generate artificial miRNAs based on the natural human miR-30 pri-miRNA transcript. For ease of cloning, we only include a minimal amount of natural miR-30 flanking sequences, while additional 5'- and 3'-sequences are derived from the mouse U6 expression vector. We have

characterized these vectors for appropriate expression and processing using small transcript northern blot, small RNA RT-PCR and RACE analyses (Boudreau *et al.*, 2008; Chen *et al.*, 2005). We recommend that newly designed miRNA shuttles be similarly defined with these techniques, since simply switching promoters, restriction enzyme sites, or expression contexts (e.g., embedding the miRNA-based stem loop in the 3'-UTR or intron of a reporter gene) can alter the pri-miRNA structure and subsequent processing.

4.2. Oligo design and cloning protocol for artificial miRNAs

The following steps are outlined in Fig. 14.3.

Step 1. Start with the artificial miRNA backbone sequence.

The miR-30-based artificial miRNA backbone consists of constant regions (5'- and 3'-flanking and loop sequences) that are partially derived from the human miR-30 pri-miRNA. The backbone is engineered with flanking restriction enzyme sites for cloning purposes. The 5'-*Xba*I and 3'-*Spe*I sites were chosen since their respective GAG and ACT nucleotides correspond to natural miR-30 stem-loop sequences. The distal (relative to the loop) single nucleotide bulge pictured in Fig. 14.1 is formed by the 5'- and 3'-flanks, while the proximal bulge consists of the 3'-end of the antisense strand; thus, the opposing nucleotide must be manipulated to maintain the bulge for proper Drosha-DGCR8 processing (see Step 3 below).

Step 2. Insert sense and antisense sequences.

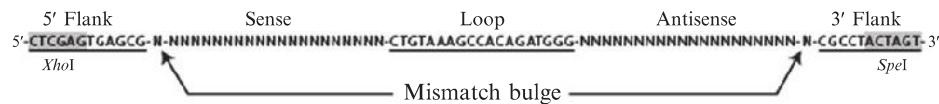
Insert the sense sequence (i.e., target site positions 1–22) immediately after the 5'-flank sequence and the antisense sequence (i.e., the reverse complement of the target site) directly after the loop sequence.

Step 3. Manipulate the 5'-bulge nucleotide to induce a mismatch.

The bulge is natural to the human miR-30 pri-miRNA transcript, and thus, we aim to maintain this structure to promote proper downstream cleavage by Drosha-DGCR8. In the example, the G nucleotide in the sense strand bulge position (5') is converted to an A nucleotide which will create a mismatch with the opposing C bulge nucleotide in the antisense region (3'). In generating artificial miRNAs harboring different siRNA sequences, the user should alter the 5'-bulge nucleotide so that it does not pair with the opposing 3'-bulge nucleotide. This can be done following these simple guidelines:

- A: if the 3'-bulge site is an A, the 5'-bulge nucleotide can be changed from T to either C, G, or A.
- C: if the 3'-bulge site is a C, the 5'-bulge nucleotide can be changed from G to either A, T, or C.

Step 1. Start with artificial miRNA backbone sequence.



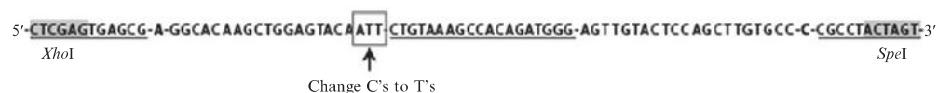
Step 2. Insert sense and antisense sequences.



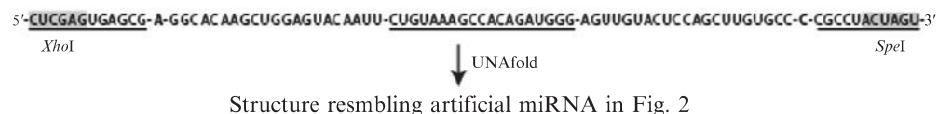
Step 3. Manipulate 5' bulge nucleotide to induce a mismatch.



Step 4. Convert C's to T's at sense 3' end to impart duplex instability.



Step 5. Convert all T's to U's and fold RNA using UNAfold



Step 6. Design and order DNA oligos.



Step 7. Anneal and polymerase extend oligos.



Step 8. PCR purification, digestion and cloning to pTOPOBluntII-U6



Figure 14.3 Artificial miRNA cloning scheme. The details for designing artificial miRNAs and cloning them into the mU6 expression vector are described in the text ([Section 4.2](#), Steps 1–8). Shown here is the example of incorporating the eGFP-targeted siRNA into an artificial miRNA scaffold.

G: if the 3'-bulge site is a G, the 5'-bulge nucleotide can be changed from C to either A or G.

T: if the 3' bulge site is a T, the 5'-bulge nucleotide can be changed from A to either C or T.

Step 4. Convert C's to T's at the sense 3'-end to impart duplex instability.

See Step 2 in shRNA cloning protocol ([Section 3.2](#)).

Note: at this point, it is good practice to ensure the hairpin sequence is void of stretches of four or more T's and any restriction enzyme sites (e.g., internal *Xho*I, *Spe*I, and *Eco*RI sites) relevant to downstream applications.

Step 5. Convert all T's to U's and fold RNA using UNAfold.

See Step 3 in shRNA cloning protocol ([Section 3.2](#)). In this case, the fold should resemble the artificial miRNA depicted in [Fig. 14.1](#).

Step 6. Design and order DNA oligos.

The artificial miRNA DNA template is made by annealing two oligos that overlap in the loop region and performing a polymerase extension reaction to create the double-stranded DNA cassette. Oligo 1 consists of the *Xho*I site, 5'-flank, sense sequence, and loop in forward orientation, while Oligo 2 is the reverse complement of the loop, antisense, 3'-flank and *Spe*I site. We add four A nucleotides to the 5'-end of each oligo; thus, Oligo 1 will begin with 5'-AAA₄ACTCGAG...-3' and Oligo 2 with 5'-AAA₄ACTAGT...-3'. These additional bases are required for efficient restriction enzyme digestion near the ends of the DNA cassette following the polymerase extension. The resulting DNA oligos can be ordered at reasonable cost from a variety of commercial vendors (e.g., Integrated DNA Technologies or Sigma-Aldrich) using the smallest synthesis scale available and standard desalting purification. Upon receipt, the oligos can be reconstituted in purified water to make a 100- μ M stock.

Step 7. Anneal and polymerase extend Oligo 1 and Oligo 2.

Reaction conditions

5 μ l 10 \times reaction buffer with MgCl₂

1 μ l Oligo 1 (100 μ M)

1 μ l Oligo 2 (100 μ M)

1 μ l dNTPs (10 mM)

0.5 μ l enzyme mix (i.e., polymerase)

41.5 μ l dH₂O

Incubate the reaction in a thermocycler:

94°C for 2 min (denaturation)

54°C for 1 min (annealing)

72°C for 15 min (extension)

Step 8. PCR purification, digestion, and cloning into Tb:mU6 plasmid

Step 8.1. Purify the extended product using a PCR Purification kit (QIAquick; Qiagen) as per the manufacturer's instructions and elute in 30 μ l water.

Step 8.2. Digest the product with *Xho*I and *Spe*I by adding the following to the 30 μ l eluate:

4 μ l 10 \times restriction enzyme buffer (NEB2; New England Biolabs)

4 μ l 10 \times BSA (10mg/ml)

1 μ l *Spe*I (10units/ μ l)

1 μ l *Xba*I (20units/ μ l)

Incubate reaction at 37°C for 4h to overnight.

- Step 8.3.** Digest 3–4 μ g of the Tb:mU6 expression plasmid with *Xba*I and *Xba*I. This plasmid contains the mouse U6 promoter followed by a multiple cloning site (MCS) and a Pol III termination signal (six T's) (Fig. 14.4). *Xba*I- (vector) and *Spe*I (artificial miRNA)-cleaved sites produce compatible sticky ends for ligation. Although there is a *Spe*I site in the Tb:mU6 MCS, we have found in prior studies that ligation to the



Figure 14.4 TOPOBluntII:mU6 (Tb:mU6) plasmid details. Tb:mU6 plasmid is ~4kb and contains a *Eco*RI-mU6 promoter–multiple cloning site–TTTTTT terminator–*Eco*RI cassette which was cloned into the TOPOBluntII vector in reverse orientation using the indicated restriction sites (*Xba*I and *Bam*HI were vector-derived, while *Nhe*I and *Bgl*II originated from the insert; all sites were destroyed upon ligation). The mU6 +1 transcription start site and Pol III terminator (TTTTTT) are shown in bold. The multiple cloning site (MCS) is underlined and contains *Kpn*I–*Pme*I–*Xho*I–*Sal*I–*Cla*I–*Hind*III–*Eco*RV–*Eco*RI–*Xma*I–*Sma*I–*Bam*HI–*Spe*I–*Xba*I–*Not*I restriction enzyme sites. Note: cloning an artificial miRNA into the *Xho*I and *Xba*I sites removes the internal *Eco*RI (in the MCS), and thus, positive clones can be screened for by *Eco*RI digestion. The relevant primer binding sites for the shRNA-tailed PCR are also shown.

*Xba*I site produces a stem loop which is more efficiently processed, yielding higher antisense RNA levels and more potent gene silencing (Boudreau *et al.*, 2008).

- Step 8.4.** Gel purify the digested fragments. We typically run the digested artificial miRNA inserts on a 2% agarose gel and excise the ~100-bp band. The digested Tb:mU6 is run on a 1% agarose gel and the ~4-kb fragment is excised. Gel extraction can be performed by various means; for example, we simply subject the gel slices to ultracentrifugation in a Spin-X column (Corning Incorporated). Precipitation may be necessary to concentrate the DNA fragments for ligation.
- Step 8.5.** Perform ligation and bacterial transformation using standard protocols. We ligate 6ng of insert to 50ng of vector and incubate at room temperature for 1h before transformation. The Tb:mU6 plasmid is kanamycin resistant, and thus, transformed bacteria should be grown on LB agar plates containing kanamycin. Because the Tb:mU6 vector is digested with two noncohesive enzymes (*Xho*I and *Xba*I), the likelihood of intramolecular vector ligation is minimal, and few, if any, kanamycin-resistant colonies grow on “vector-only” control plates. If necessary, the vector can be treated with alkaline phosphatase after restriction enzyme digestion to further minimize the potential for background colonies. We typically pick four to six colonies per artificial miRNA construct and grow each of them overnight in 3ml liquid LB cultures containing kanamycin. The following day, minipreps are performed using the Qiaprep kit which yields plasmid DNA of sufficient quality for cell culture applications.
- Step 8.6.** Screen for positive clones by *Eco*RI digestion and gel electrophoresis. Tb:mU6 vector-only plasmid will yield ~3.5kb, ~380bp, and ~80bp fragments indicating a negative clone; we recommend digesting the parental Tb:mU6 vector alongside the potential artificial miRNA clones to serve as a reference. The positive constructs with successful insertion of an artificial miRNA will yield ~3.5kb and ~500bp bands.
- Step 8.7.** Sequence positive clones using the M13 reverse primer. Refer to the information regarding sequencing of shRNAs (Section 3.2—Step 6.8).

4.3. Materials for artificial miRNA cloning

Tb:mU6 plasmid (expression vector)
Overlapping DNA oligos for artificial miRNA
Expand high-Fidelity Polymerase and buffers (Roche)
Restriction enzymes and buffers (*Xba*I, *Spe*I, *Xba*I, and *Eco*RI)
Gel electrophoresis equipment and reagents
Gel extraction kit or Spin-X columns (Corning)
DNA ligase and buffer
Chemical competent bacterial cells
Kanamycin-containing LB agar growth plates
Qiaprep MIniprep kit (Qiagen)



5. SCREENING RNAI VECTORS FOR SILENCING EFFICACY *IN VITRO*

Although we select siRNA sequences based on the most significant determinants of gene silencing efficacy (i.e., strand biasing and GC content), not all sequences will be functional. Thus, we typically generate several constructs, each with unique sequences, for a given target gene. These constructs must then be screened for gene silencing efficacy to identify those which mediate silencing levels suited to the researchers' needs. Gene silencing can be assessed at the mRNA level by performing real-time quantitative PCR (preferred) and/or the protein level via Western blot analysis (dependent upon protein half-life) following delivery of the RNAi vectors into cells. For practical purposes, RNAi efficacy screens are generally carried out in highly transfectable cultured cell lines. With this approach, the target mRNA may be expressed endogenously or from cotransfected plasmids (Boudreau *et al.*, 2008; Paul *et al.*, 2002; Sui *et al.*, 2002). Screening against exogenously supplied targets (e.g., cotransfected target-reporter expression plasmids) in highly transfectable cell lines is particularly useful if the target is only expressed in poorly transfectable cells (e.g., primary neurons). In addition, silencing of coexpressed targets is more efficient since most cells receive both RNAi and target expression plasmids due to the nature of transfection, and target expression levels can be readily controlled. Conversely, silencing of endogenous mRNAs may be limited by transfection efficiency, particularly when the treated population is analyzed as a whole. For larger-scale screens, the use of target-reporter fusions (e.g., luciferase or fluorescent) may expedite the process of narrowing candidates (Mousses *et al.*, 2003). Silencing of the natural target can be subsequently confirmed with other functional, biological, and biochemical assays.

5.1. Screening against a cotransfected reporter constructs

For screening RNAi expression vectors *in vitro*, we often use HEK293 cells because they are easy to culture and highly transfectable with liposome-based reagents (e.g., Lipofectamine 2000, Invitrogen). Prior studies validate that siRNA, shRNA, and artificial miRNA vectors are capable of effective gene silencing in HEK293 cells, supporting that the necessary RNAi machinery is present. In our example, we test the eGFP-targeted artificial miRNA (miGFP) for its ability to silence GFP expression from a cotransfected reporter plasmid (Fig. 14.5). For this experiment, we seeded 200,000 cells per well in a 24-well culture plate. The next day, cells were cotransfected in triplicate with 75 ng of GFP expression plasmid along with one of the following plasmids: (1) Tb:mU6, serving as the promoter-only control; (2) Tb:mU6-miSCA1, an artificial miRNA targeting ataxin-1, providing a nontargeted RNAi control; and (3) Tb:mU6-miGFP, the on-target RNAi construct in this study. Twenty-four to forty-eight hours posttransfection, we evaluated GFP levels by fluorescent microscopy and observed significant knockdown in miGFP-treated cells, relative to the controls.

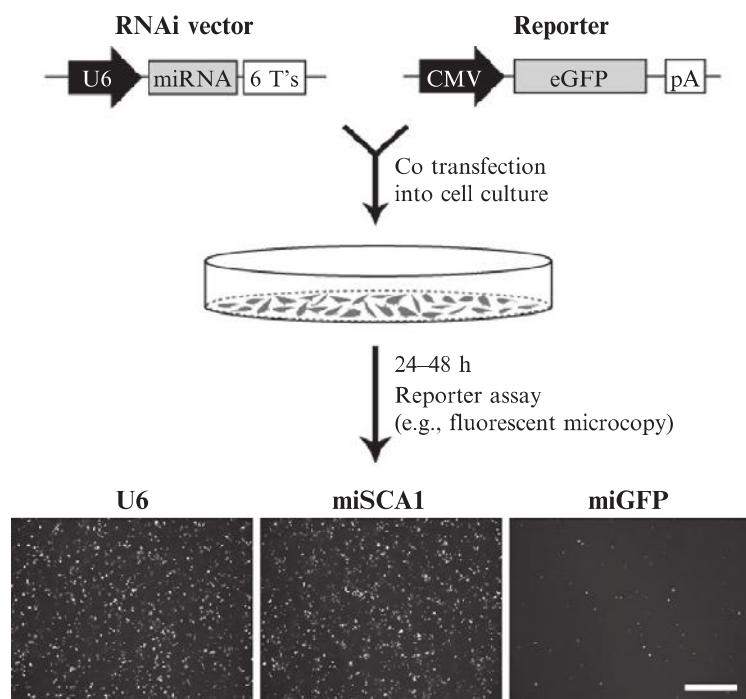


Figure 14.5 Example silencing experiment targeting an eGFP reporter. Plasmids expressing U6-driven artificial miRNAs or a CMV-driven eGFP reporter were cotransfected into HEK293 cells with a ratio of four RNAi:one eGFP (300ng:75ng). After 48 h, fluorescence microscopy was performed to evaluate eGFP levels. Representative photomicrographs of eGFP autofluorescence show evident silencing of eGFP expression in miGFP-treated cells, relative to the controls (U6 promoter-only and miSCA1, a nontargeted RNAi control). Scale bar=1 mm.

Additional information and recommendations for screening by cotransfection:

1. A variety of reporter options are available. The user may clone their target transcript into fluorescent or luciferase (psiCheck2, Promega) reporter systems, or as an epitope-tagged version for Western blotting.
2. We recommend master mixing the target reporter prior to distributing among the various treatments to ensure consistency across treatments. In our experience with 24-well plates, we typically use 25–100 ng of fluorescent reporter plasmids and 5–20 ng of luciferase-based reporters.
3. A dose response should be performed to further evaluate the potency of the RNAi constructs. This is important when multiple RNAi constructs show maximal silencing in a given experiment, supporting the need to test lower doses to identify the most efficacious sequence. In our example, we tested a RNAi:reporter ratio of 4:1. We recommend testing a 1:1 ratio as well. In the low-dose condition, supplementing empty Tb:mU6 plasmid to balance total RNAi plasmid amounts, relative to the high-dose condition, is a good practice.
4. Results from reporter assays are generally translatable to the natural transcripts; however, we advise that target silencing be confirmed in the intended experimental setting before downstream effects are queried or long-term studies are initiated.

5.2. Screening against endogenously expressed targets

Silencing of natural targets can be directly assessed if the target gene is expressed in a highly transfectable cell line (e.g., HEK293, HeLa, or NIH3T3, among others) of the relevant species. Previous studies from our laboratory have demonstrated silencing of endogenously expressed transcripts (e.g., encoding huntingtin or ataxin-1) in HEK293s and C2C12 cells ([Boudreau et al., 2008, 2009b](#); [McBride et al., 2008](#)). In these experiments, 200–400 ng of RNAi expression plasmids were transfected into cells grown in 24-well plates, and gene silencing was evaluated 24–48 h later by QPCR or Western blot analyses measuring mRNA and protein levels, respectively. The methods for these experiments are not described here but can be found in the relevant references.



6. INTEGRATION INTO VIRAL VECTORS

Upon identifying effective hairpins, the RNAi expression cassettes can be easily subcloned into various viral vector systems for downstream applications. The University of Iowa Gene Transfer Vector Cora facility offers

an assortment of plasmids for production of recombinant adenovirus, adeno-associated virus (AAV) and lentivirus (FIV). The mU6-driven artificial miRNA and shRNA expression cassettes described above are flanked by *Eco*RI sites and can be easily cloned into compatible sites (*Eco*RI or *Mfe*I) in each of these viral vector platforms. AAV vectors have become a powerful tool for *in vivo* application and, with several capsids available, offer a broad capacity to transduce many different cell types and tissues. To date, AAV-based RNAi vectors have been successful in achieving gene silencing in a variety of tissues including muscle, liver, and brain, among others (Davidson and McCray, 2011). As a note of caution, AAV-shRNA vectors have been shown to cause toxicity in mouse liver and brain due to high-level shRNA expression in conjunction with such a robust delivery platform (Boudreau *et al.*, 2009a; Grimm *et al.*, 2006; Martin *et al.*, 2011; McBride *et al.*, 2008). Thus, we recommend expressing artificial miRNAs, which are expressed at lower levels relative to shRNAs, from AAVs to achieve more tolerable RNAi expression levels *in vivo*. High-level shRNA expression is more advantageous for low-copy applications, such as generating stable cell lines with integrating lentiviral vectors, where single-copy insertions are often desired. In this instance, shRNAs may be better suited than miRNA-based vectors, where low-level expression may preclude sufficient silencing. These examples highlight the importance of considering the balance of efficacy and toxicity when selecting the most suitable RNAi expression strategy and delivery platform.



7. SUMMARY

RNAi triggers expressed from vector-based systems provide important tools for experimental biology and over the past decade have become a focus for therapeutic development. This chapter provides the reader with a stepwise protocol for generating shRNA- or artificial miRNA-based systems for use in gene silencing experiments. In addition, we describe methods for introducing these RNAi expression systems into viral vectors for *in vivo* applications, providing a powerful approach to query gene function or validate drug targets.

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