Chapter 2

Rapid Cloning and Validation of MicroRNA Shuttle Vectors: A Practical Guide

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Abstract

MicroRNAs (miRNAs) have emerged as important modulators of eukaryotic gene expression through a process called RNA interference (RNAi). Over the last several years, a large amount of work has focused on understanding how miRNAs are expressed and processed to a biologically functional form. This knowledge has enabled the development of RNAi as a molecular tool for investigating basic biological questions or as a therapeutic technique.

Artificial miRNA shuttle vectors can be engineered to mimic natural miRNAs and subsequently used to suppress any gene of interest. Here, we describe a simple method to build and functionally validate artificial miRNA shuttles.

Key words: RNAi, MicroRNA, miRNA, siRNA, Inhibitory RNA, Gene silencing, Gene therapy

1. Introduction

RNA interference (RNAi) has emerged as an important modulator of gene expression in eukaryotic cells (1, 2). RNAi refers to post-transcriptional control of gene expression mediated by small non-coding microRNAs (miRNAs), which are naturally occurring transcripts encoded in the genomes of a variety of organisms ranging in complexity from single-celled algae to humans (3–7). Functionally, miRNAs reduce protein expression by directing cellular gene silencing machinery to specific target messenger RNAs (mRNAs), thereby inhibiting their translation or inducing transcript decay. Specificity is accomplished by base-pairing between miRNAs and complementary sequences on target mRNAs (3, 4, 8, 9). Over the last several years, numerous studies have described

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the cellular pathways controlling miRNA biogenesis and gene silencing (3, 10–23). An important consequence of this growing knowledge is the development of RNAi as a molecular tool to investigate basic biological questions, or as a therapeutic (24).

2. miRNA Biogenesis

Artificial miRNAs, and other small inhibitory RNAs that mimic natural miRNA structures, can be engineered to silence potentially any gene of interest. It is therefore helpful to understand natural miRNA biogenesis when developing RNAi as a tool or a therapeutic (Fig. 1).

In cells, miRNAs are first transcribed from the genome as primary miRNA transcripts (pri-miRNAs), which form intramolecular hairpin (i.e., stem-loop) structures (10, 11, 17, 25–28). Subsequently, pri-miRNAs undergo a series of processing events, catalyzed by several proteins, to generate the mature miRNA, which is typically 19–25 nucleotides (nt) long. First, in the nucleus, sequence and structural elements in the pri-miRNA transcript direct a microprocessor complex containing Drosha and DGCR8 proteins to cleave the RNA at a specific location (16, 22, 25). DGCR8 binds the pri-miRNA and serves to position Drosha correctly at the stem base, where it then makes a staggered cut, producing a shorter (~65–70 nt) pre-miRNA hairpin containing a di-nucleotide 3′ overhang. The nuclear export factor, Exportin-5, then binds the 3′ overhang and transports the pre-miRNA through the nuclear pore (23). In the cytoplasm, the ribonuclease Dicer then binds the 3′ overhang and cleaves the pre-miRNA two RNA helical turns away (approximately 21 nt) (13, 14). This cut removes the loop region and produces the mature duplex miRNA containing di-nucleotide 3′ overhangs at both ends. One strand (the antisense "guide" strand) is then incorporated into a riboprotein multimer called the RNA-induced silencing complex (RISC) (9, 21). This miRNA-loaded RISC is ultimately responsible for sequence-specific gene silencing of target mRNAs (15, 20). The sense (or "passenger") strand of the miRNA may be degraded or used to guide a second RISC. Thus, in some cases, both miRNA duplex strands can direct gene silencing. There are two major mechanisms by which RISC mediates gene silencing: translational inhibition and mRNA destabilization or target transcript cleavage. The mechanism of repression is determined by the degree of nucleotide sequence complementarity between the miRNA guide strand and the target mRNA, and each pathway is associated with different RISC components. In general, most miRNAs are only partially complementary to the target mRNAs they control. Indeed, for some miRNA:mRNA interactions, only 7 nt of

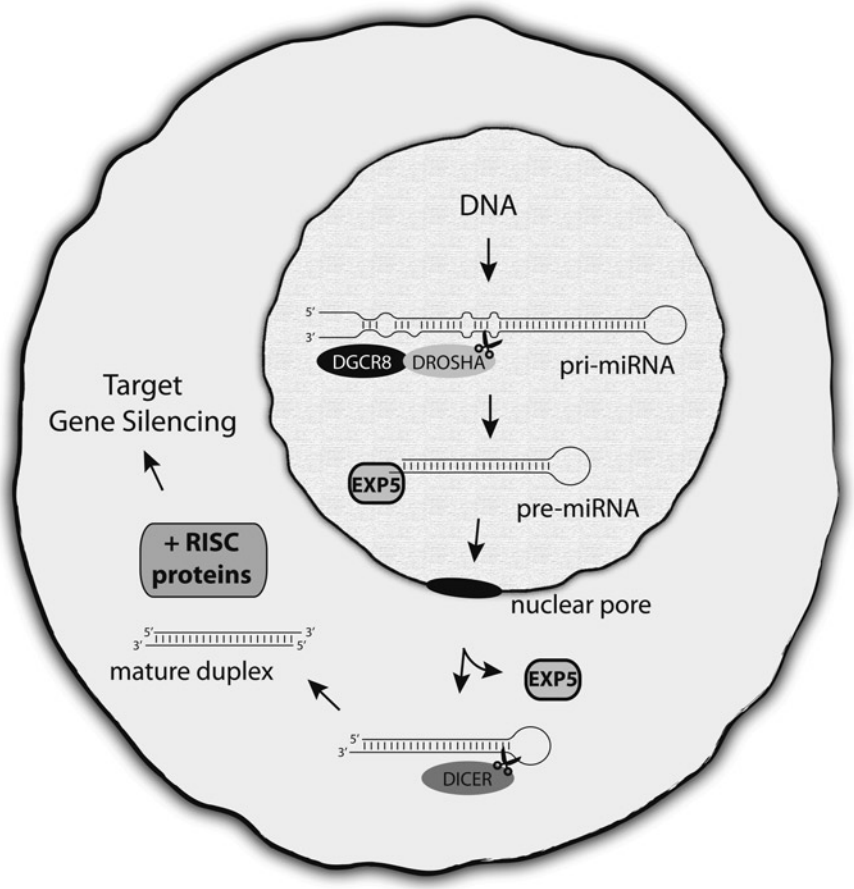

Fig. 1. MiRNA biogenesis pathway. Natural miRNAs are first transcribed from genomic DNA as primary miRNA transcripts (pri-miRNAs), which form hairpin structures. In the nucleus, the pri-miRNA is cleaved at one end by the Microprocessor complex, which contains Drosha and DGCR8 proteins. This cut produces a shorter pre-miRNA hairpin containing a two nucleotide 3′ overhangs. Exportin-5 (EXP5) then shuttles the pre-miRNA to the cytoplasm through the nuclear pore. There, a second cut, mediated by the Dicer enzyme, removes the loop region to produce a mature duplex miRNA containing di-nucleotide 3′ overhangs at both ends. One strand (the antisense "guide" strand) incorporates into a riboprotein multimer called the RNA-induced silencing complex (RISC), which then directs sequence-specific gene silencing of target messenger RNAs. Artificial miRNAs are designed to enter the pathway as pri-miRNAs in the nucleus and, therefore, undergo all processing steps required to produce a mature miRNA.

homology (miRNA nucleotides 2–8; called the "seed region") is sufficient to cause gene silencing (8). This incomplete pairing was initially thought to occur primarily through a translational inhibition mechanism that resulted in reduced protein levels, while target mRNA abundance remained unaffected. However, recent evidence supports that incomplete miRNA base pairing also causes target mRNA destabilization (29). Perfect or near-perfect miRNA:mRNA complementarity across the ~19–25 nt stretch of homology typically induces the more robust cleavage-based silencing mechanism (12). The latter is sometimes referred to as an "siRNA" mechanism (see next paragraph for details).

3. Artificial Inhibitory RNAs

Artificial inhibitory RNAs can be designed to reduce the expression of any gene of interest. There are three major classes, each of which mimics the structure of natural miRNAs at different stages of processing. The first class, small interfering RNAs (siRNAs), are structurally similar to mature miRNA duplexes (12), usually with the antisense strand exhibiting perfect complementarity to the intended target mRNA. In various publications, siRNAs may also be referred to as short interfering RNAs, small inhibitory RNAs, or short inhibitory RNAs. SiRNAs are synthesized in vitro, and upon delivery to cells, require no maturation by miRNA biogenesis machinery, although some longer siRNAs may be processed by Dicer (30). The second class of artificial inhibitory RNAs is short hairpin RNAs (shRNAs), which are structurally similar to pre-miRNAs (7, 31). Typically, shRNAs are designed with ~21-nt paired sense and antisense sequences connected by an unpaired loop that is often derived from natural miRNA sequences. shRNAs are most often expressed intracellularly from DNA expression cassettes and are not cleaved by Drosha. Instead, the transcription start and stop sites are positioned to generate shRNAs with ~2–3-nt 3′ overhangs, thus making them substrates for nuclear export and subsequent processing by Dicer which releases small RNA duplexes functionally equivalent to siRNAs. The third class of artificial inhibitory RNAs, miRNA shuttles (aka artificial miRNAs), mimic pri-miRNAs (11, 27, 28). Like shRNAs, miRNA shuttles are expressed intracellularly from DNA transgenes. miRNA shuttles typically contain natural miRNA sequences required to direct correct processing, but the natural, mature miRNA duplex in the stem is replaced by sequences specific for the intended target transcript (Fig. 2). Following expression, an artificial miRNA is cleaved by Drosha and Dicer to release this embedded siRNA-like region.

Each class of artificial inhibitory RNAs is capable of mediating gene silencing despite entering the pathway at different steps. The main differences between siRNAs and shRNAs or miRNA shuttles are the mode and duration of expression and gene silencing. As noted, siRNAs are chemically synthesized and must be delivered to target cells using liposomes or other nucleic acid delivery vehicles. Moreover, siRNAs have relatively short half-lives in vivo (32). As a result, a single siRNA administration produces only transient gene silencing effects, and long-term target gene suppression requires chronic or repeated siRNA delivery. On the contrary, shRNAs and miRNAs are expressed in vivo from DNA-based delivery systems, such as viral vectors or plasmids, which may be capable of achieving long-term expression and gene silencing after only one administration (33).

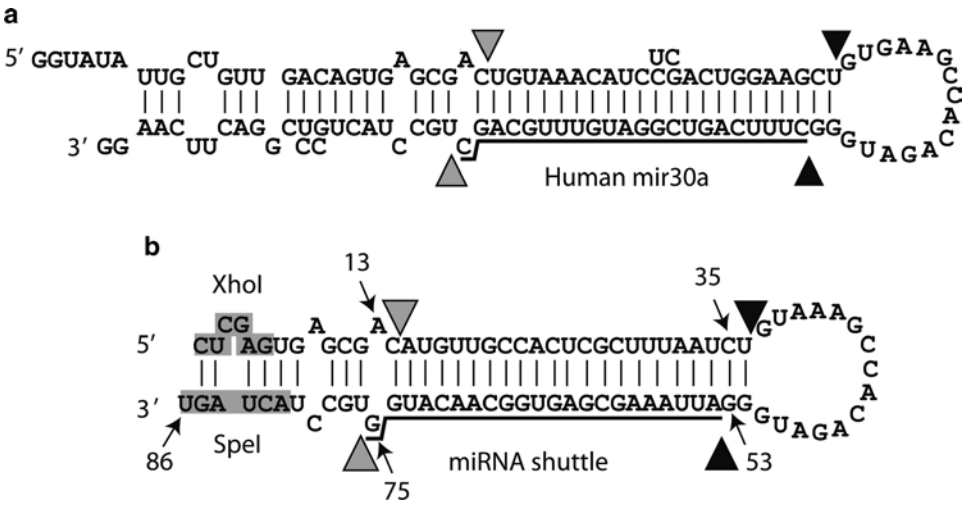

Fig. 2. Artificial miRNA shuttles derived from natural human mir-30a. (**a**) Sequence and structure of human mir-30a. (**b**) An artificial miRNA shuttle, based on human mir-30a, targeting the *E. coli* LacZ gene. *Gray* and *black arrowheads* indicate Drosha and Dicer cut sites, respectively. The mature guide strand is underlined. In (**b**), *Xho*I and *Spe*I cloning sites are *boxed*. Further details are provided in the text. The G at position 39 in the loop sequence is changed to an A in the miRNA shuttle. This changes facilitates folding in Unafold, but it does not affect function.

4. Advantages of miRNA Shuttles

shRNAs were the first generation of expressed artificial inhibitory RNAs applied in vivo. Although several reports demonstrated efficient shRNA-mediated gene silencing (34–36), recent evidence supports that miRNA shuttles are more predictably processed, efficient, and safer than shRNAs (37–40). Here, we discuss the advantages of using miRNA shuttles in RNAi expression vectors, instead of shRNAs.

As previously mentioned, shRNAs and miRNAs are typically expressed in vivo from DNA expression cassettes but differ in processing events required for maturation. This differential processing has direct implications for the predictability of the final product. Drosha cleaves miRNA shuttles but not shRNAs. This is important because the Drosha cut site directs Dicer binding and cleavage, and together these two events ultimately determine the sequence of the mature guide strand. Thus, because engineered miRNA shuttles faithfully recapitulate miRNA structures required for Drosha and Dicer cleavage, the guide strand is more consistently produced and accurately predicted (28, 37). In contrast, the transcription start and termination sites define shRNA 5′ and 3′ ends, respectively (7, 31). In a given transcript, these can vary by a few nucleotides, which results in a mixed population of mature products. Thus, shRNA-derived guide strands may be more variable than

similar sequences produced from an miRNA-based delivery system. Because sequence specificity is a hallmark of RNAi, using a system with the most accurately predictable final product is desirable.

The differential processing of miRNA shuttles and shRNAs also affects the methods by which they are expressed. ShRNAs must be positioned near the transcription start and stop sites to ensure proper maturation and, ultimately, gene silencing function. Such requirements limit the options available for shRNA expression to systems with well-defined transcription start and stop sequences. Indeed, shRNAs are most typically expressed from RNA pol III-dependent promoters that have strong ubiquitous and constitutive expression patterns. In contrast, because Drosha cleavage, not transcription, defines the critical Exportin-5 and Drosha-binding site, miRNA shuttles can be expressed from any RNA pol II- or pol III-dependent promoter, which broadens their usage for tissue-specific and/or inducible expression applications (17, 28, 37, 41–44). Additionally, miRNA shuttles can be embedded in introns or untranslated regions (UTRs) of coding genes, which allows for co-expression of a reporter gene and an miRNA from the same transcript (44, 45). On the contrary, due to their expression restrictions, shRNAs are not amenable to such strategies.

Another notable difference between shRNAs and miRNA shuttles is that the former may be more prone to inducing nonspecific toxicity. Although several studies have shown shRNAs to direct in vivo gene silencing effectively with no overt toxicity, recent reports have raised safety concerns that are likely related to high-level, uncontrolled shRNA expression which may cause nonspecific gene dysregulation by disrupting endogenous miRNA biogenesis (36, 39, 40). In one study, vector-delivered shRNA expression in mouse brain caused robust toxicity in striatal neurons (40). However, expressing the identical mature inhibitory RNA sequence in the context of an miRNA shuttle system driven by the same promoter mitigated this toxicity while still directing efficient target gene silencing (40). This improved safety profile likely resulted from the miRNA shuttles being expressed at lower levels compared to analogous shRNAs. Although the reasons for these expression differences remain unclear, miRNA shuttles more closely mimic natural miRNA structures and may be more efficiently processed and less likely to interfere with endogenous miRNA biogenesis and function.

In summary, miRNA shuttles may be superior to shRNAs because they more closely recapitulate natural miRNA structures, are more predictably processed, are amenable to control by tissue-specific and/or regulated promoters, can be co-expressed from coding gene introns or UTRs, and may have a better safety profile. For these reasons, miRNA shuttles are outstanding systems for long-term RNAi studies. In this chapter, we present a rapid method to design and clone miRNA shuttles.

5. Design of miRNA Shuttles

Our miRNA shuttle cloning system is modified from a human mir-30a (hsa-mir-30a)-derived strategy originally described by Zeng and Cullen (28, 46). The natural pri-mir-30 reference sequence is pictured, and the mature antisense mir-30 miRNA produced following Drosha and Dicer cleavage is 22 nt in size (Fig. 2a). Our pri-miRNA shuttles are derived from this sequence with the following modifications: (1) the mature mir-30 duplex sequences are replaced by sequences corresponding to the gene of interest, (2) the pri-miRNA shuttle sequences are slightly shorter than the reference sequence, and (3) *Xho*I and *Spe*I restriction enzyme sites are added for cloning purposes (Fig. 2, boxed gray). Some natural mir-30 features are maintained, including Drosha and Dicer cut sites (Fig. 2, gray and black arrowheads, respectively) and the unpaired loop sequence. In addition, the mismatch located just upstream of the Drosha cut sites should be maintained for proper processing (Fig. 2, arrows). Here, we describe our method to generate the miRNA shuttle shown in Fig. 2, which targets the *Escherichia coli* LacZ transcript. For determining which target sequences to embed in the miRNA shuttles, we employ the currently available siRNA "design rules" (15, 20, 47, 48). Even yet, not all inhibitory RNAs are functionally equivalent and it is difficult to predict which sequence will most effectively elicit target gene silencing. We therefore typically design four to five artificial miRNAs per target gene to improve the chance of generating a highly effective inhibitory RNA.

For all steps below, refer to Figs. 3 and 4.

Step 1: Choose 22-nt mature miRNA sequences

There are several publications describing specific rules for optimal inhibitory RNA design (15, 20, 47, 48). Among these, we incorporate three in our miRNA shuttles. First, RISC preferentially loads the more thermodynamically unstable (i.e., more AU-rich) 5′ end of an miRNA duplex. Thus, to ensure proper loading of the antisense guide strand, the target site sequence should consist of a GC-rich 5′ end and an AU-rich 3′ end (last 3–4 bases). Strand-biasing primarily depends on the "core" duplex region depicted in Fig. 2. The second rule is to select sequences that will yield an antisense guide strand with ~30–60% GC content, the optimal range for potent RNAi-mediated gene silencing. Finally, if using a RNA pol III-based promoter, such as the example described in this method, ensure that there are no long stretches (four or more) of tandem T (U in RNA) nucleotides within the mature miRNA shuttle, since pol III terminates on ~4–5 or greater T's. Long

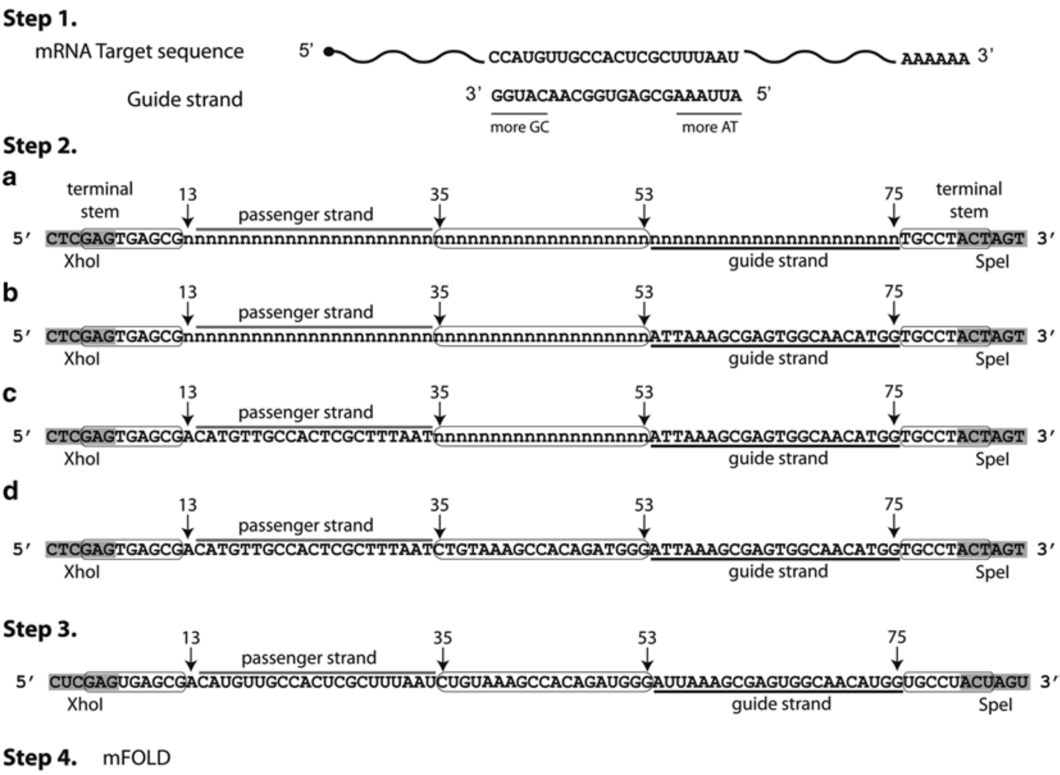

Fig. 3. Designing and cloning miRNA shuttle vectors: steps 1–4. All details are described in the text.

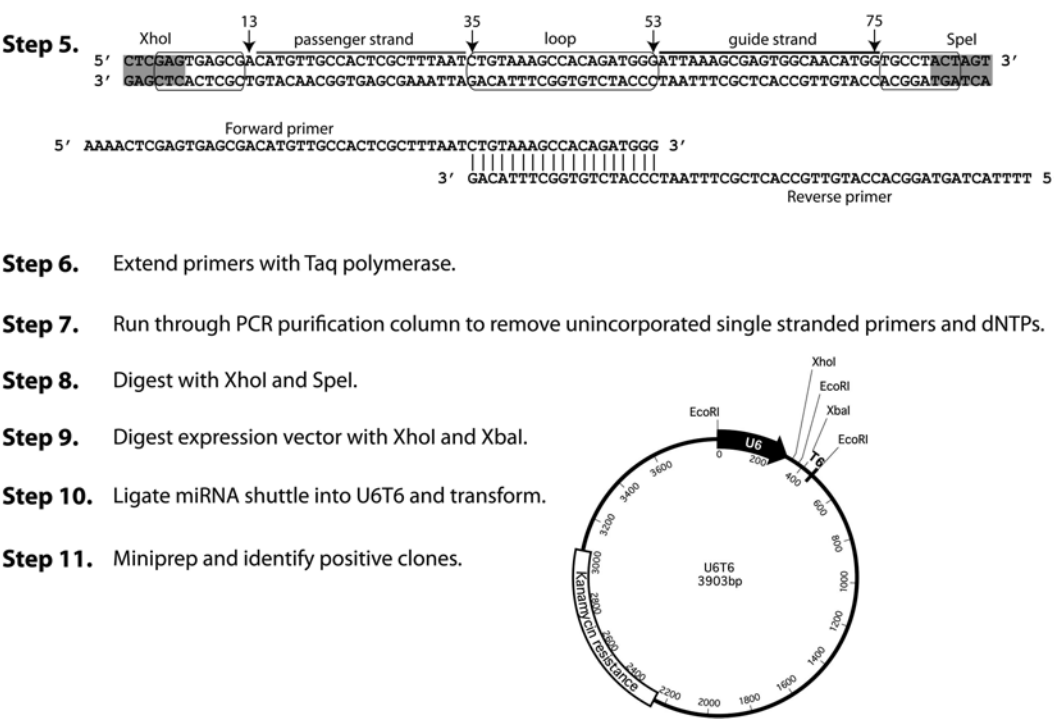

Fig. 4. Designing and cloning miRNA shuttle vectors: steps 5–11. All details are described in the text.

stretches of T's will not effect miRNA expression if using a pol II-based promoter, which terminates with a typical poly-adenylation signal (AATAAA). In this example, a 22-nt sequence from the *E. coli* LacZ gene is embedded in the miRNA backbone. The guide strand is the reverse complement and forms perfect Watson–Crick base pairing with the cognate target.

Step 2: Generate DNA template reference sequences and corresponding miRNA shuttle.

We generate miRNA shuttles using DNA polymerase extension of two annealed DNA oligonucleotide primers designed to contain all required sequences shown in Fig. 2. The natural mature miRNA duplex, in this case from hsa-mir-30a, is replaced with the target gene-derived artificial miRNA duplex (Fig. 2). Regardless of the mature miRNA sequence, each miRNA and, therefore, each oligonucleotide, contains common sequence elements so that the cloning method is identical for every miRNA shuttle generated. As a result, primer design is critical for proper miRNA production and cloning. To aid primer design, it is helpful to generate a reference sequence for the DNA transcription template used to produce a given miRNA shuttle (Figs. 2 and 3).

(A) The terminal stem (nucleotides 1–12 and 76–86).

Nucleotides 1–12

1. The 5′ end (nucleotides 1–6) contains an XhoI cloning site (5′ CTCGAG 3′), of which the 3′ GAG nucleotides are derived from natural mir-30a sequences.
2. Additional mir-30a-derived stem sequences located at nucleotide positions 7–12 (5′ TGAGCG 3′) are included in every mir-30a shuttle.

Nucleotides 76–86

1. The T nucleotide at position 76 is immediately 3′ of the Drosha cut site on the antisense strand.
2. Positions 76–83 (5′ TGCCTACT 3′) are mir-30a-derived stem sequences in which the 3′ terminal ACT nucleotides are the first half of a *Spe*I restriction site.
3. The AGT nucleotides located at positions 84–86 are added to generate a 3′ terminal *Spe*I site (5′ ACTAGT 3′).

(B) The artificial miRNA guide strand (nucleotides 54–75).

1. This is the exact guide strand sequence shown in step 1.
2. The guide strand is placed between the Dicer and Drosha cut sites located at positions 53 and 76 (Fig. 2, black and gray arrowheads, respectively, and Fig. 3, step 2B).

(C) The artificial miRNA passenger strand (nucleotides 13–34).

1. The first nucleotide of the passenger strand (position 13) must be mismatched with position 75 of the guide strand to best mimic mir-30a structure.
   (a) In our example, note the last guide strand nucleotide at position 75 is a G.
   (b) The mismatch at position 13 must then be an A or G, since A:U is a normal Watson–Crick base pair. The natural has-mir-30a transcript has an A:C mismatch, but any mismatch will work at this position.
   (c) If the position 75 nucleotide is a C, position 13 must be a U (T in DNA template), C, or A.
   (d) If the position 75 nucleotide is a U (T in DNA template), position 13 must be a U or C.
   (e) If the position 75 nucleotide is an A, position 13 must be an A, G, or C.
2. The remaining 21 nucleotides of the passenger strand are identical to positions 2–22 of the mRNA target sequence.

(D) Loop sequence (nucleotides 35–53).

1. The loop sequence (5′ – CTGTAAAGCCACAGATGGG – 3′) is derived from hsa-mir-30a, with a single modification that facilitates folding in UNAFOLD (see below) but does not affect function.
2. The two terminal nucleotides (underlined here) at both ends base pair and form a portion of the miRNA shuttle stem (5′ – $\underline{CT}_{36}$GTAAAGCCACAGATG$\underline{G}_{52}\underline{G}$ – 3′), but all other nucleotides remain unpaired (Figs. 2 and 3). Note again that T_{36} is a U in the miRNA transcript, which allows base-pairing with G_{52}.

Steps 3 and 4. Convert the DNA transcription template from step 2 into an RNA molecule (replace T's with U's) and then confirm secondary structure using UNAFOLD. Among the many free online RNA folding programs available, we have found the http://www.idtdna.com/Scitools/Applications/unafold/ website to be particularly quick and user friendly. The UNAFOLD program typically identifies several predicted secondary structures. If correctly designed, the top predicted structure for each miRNA shuttle should have the same features as that shown in Fig. 2b. The only variance between two different miRNA shuttles will be the duplex miRNA portion. Identify Dicer and Drosha cut sites (Fig. 2b) to confirm that the expected guide strand will be excised and that the "core" duplex agrees with strand-biasing rules.

6. Primer Design and Cloning of miRNA Shuttles

Step 5: Design primers

After the reference sequences for each miRNA shuttle have been generated, the next step is to design DNA oligonucleotide primers to build the miRNA transcription templates. Two primers are required per miRNA shuttle (Fig. 4). As shown, add four nucleotides to the 5′ end of each oligonucleotide to provide some additional sequences for efficient *Xho*I and *Spe*I restriction enzyme digestion required in step 8. The forward and reverse primers overlap at the loop sequence, and the unpaired sequences in each primer constitute the miRNA shuttle stem. There are no special oligonucleotide purification requirements; primers can be ordered at low cost from any commercial vendor using the smallest synthesis scale. Standard desalting provides sufficient purity and no additional purification steps, such as PAGE isolation, are required. Reconstitute primers at 1 μg/μl concentrations in purified water.

Step 6: Generate full-length miRNA shuttles using Taq DNA polymerase.

The annealed loop portion of the forward and reverse primers (see step 2, D1 above) is predicted to form an intramolecular hairpin and self-duplex at 37°C, which could impede the efficiency of the primer extension reaction. However, this natural mir-30 loop sequence has a predicted melting temperature (T_m) of 56.7°C, which allows us to minimize these potential intramolecular interactions by performing primer extensions at high temperatures using thermostable Taq polymerase as follows:

5 μl 10× Taq polymerase buffer (Bioline)

1 μl forward primer (1 μg/μl)

1 μl reverse primer (1 μg/μl)

4 μl 2.5 mM dNTPs

1 μl 50 mM $MgCl_2$

0.5 μl Taq polymerase (5 units/μl)

37.5 μl dH_2O

Incubate the reaction at the following temperatures for one cycle:

94°C for 2 min; 52°C for 1 min; 72°C for 15 min.

Step 7: Purify product using a PCR Purification kit (QIAquick; Qiagen) and elute in 30 μl water.

Step 8: Digest the linear DNA transcription template with *Xho*I and *Spe*I:

30 μl eluant

4 µl 10× restriction enzyme buffer (NEB2; New England BioLabs)

1.0 µl *Spe*I (10 units/µl)

1.0 µl *Xho*I (20 units/µl)

4 µl 10× BSA (10 mg/ml)

Incubate at 37°C for 4 h to overnight

Step 9: Digest U6T6 vector with *Xho*I and *Xba*I, and gel purify the digested vector and miRNAs (from step 9).

Step 10: Ligate miRNA shuttle into U6T6.

To produce an miRNA transcript, the DNA transcription template generated in steps 1–7 must be cloned downstream of a promoter and termination signal. In this example, we use the RNA polymerase III-dependent mouse U6 promoter to transcribe miRNA shuttles. Downstream of the U6 promoter is a pol III termination signal (TTTTTT). The *Xho*I–*Spe*I digested product from step 7 is cloned between the U6 promoter and the termination signal using *Xho*I–*Xba*I restriction sites. *Spe*I and *Xba*I digested ends are compatible. An obvious question is, "why not include *Xba*I instead of *Spe*I at the 3′ end of the miRNA template?" Theoretically, and in practice, *Xba*I–*Xba*I ligation produces functional miRNAs. However, we have found that *Spe*I–*Xba*I ligation produces a more robust miRNA shuttle expression and gene silencing compared to identical miRNAs cloned using only *Xba*I. The reason for this difference remains unclear, but may be due to slight secondary structure changes that alter the pri-miRNA transcript stability and/or processing, therefore affecting the gene silencing efficiency. Thus, the choice of restriction enzymes and nearby flanking sequences can impact the overall functionality of the resulting miRNA shuttle.

Following digestion, gel purify and quantify the miRNA and U6T6 products, then ligate and transform into bacteria using standard protocols. U6T6 is kanamycin resistant and colonies should, therefore, be screened on LB agar plates containing kanamycin. Because the U6T6 vector is digested with two non-cohesive enzymes (*Xho*I and *Xba*I), no intramolecular ligation should occur, and we typically did not find kanamycin resistant colonies on "vector-only" control plates. To further minimize the potential for background, the vector should be treated with alkaline phosphatase after digestion. If little background is evident, we typically pick four colonies per miRNA shuttle construct and grow each of them in liquid culture for DNA miniprep (using QIAquick; Qiagen, for transfection quality plasmid).

Step 11A: Identify positive miRNA shuttle clones.

Positive plasmids can be identified by *Eco*RI restriction enzyme digestion, followed by agarose gel electrophoresis (1.5% TBE or TAE gel) and UV imaging of ethidium bromide-stained DNA. Empty U6T6 has three *Eco*RI sites; one is lost upon *Xho*I–*Xba*I digestion (Fig. 4, step 8).

Expected *Eco*RI products for successfully cloned U6T6 miRNA expression cassettes:

Empty U6T6	U6T6 with miRNA shuttle
3,437 bp (contains vector)	3,437 bp (contains vector)
383 bp (contains U6 promoter)	489 bp (contains entire U6 miRNA cassette)
83 bp (contains T6 terminator)	

Step 11B: Sequence U6 miRNA plasmids containing the correct *Eco*RI digestion pattern.

Sequence two positive clones from each miRNA expression cassette to ensure that errors in primer synthesis or extension did not occur. We use ABI dye terminator sequencing and an ABI 3130xl sequencer (Applied Biosystems). Sequencing reactions of some miRNA hairpin plasmids prematurely terminate because of strong secondary structures. To prevent this, include 4% dimethyl sulfoxide (DMSO) in the sequencing reaction.

7. Rapid Screen to Identify Functional miRNA Shuttles

As mentioned, although the siRNA "design rules" are good guidelines for miRNA construction, it is difficult to predict which sequence will most effectively direct target gene silencing, if at all. Functional validation is therefore required. Typically, miRNA shuttle plasmids are transfected into a cell line expressing the target gene of interest naturally or from an exogenously delivered expression plasmid. If an easily transfected cell line expressing your gene of interest is available, perhaps the easiest method to determine gene silencing efficacy is to measure target gene expression using standard biochemical or molecular techniques such as western blotting or real-time PCR, 1 or 2 days post-transfection. If easily transfected cell lines expressing your gene of interest are not available, another option is to use the dual luciferase assay system we describe here. This method circumvents transfection-related inconsistencies, which can affect identification of effective miRNA shuttles. This system, modified from a commercial vector, provides rapid, consistent, and quantitative measurement of

gene silencing, regardless of transfection efficiency, due mostly to an internal transfection control. Our method requires (1) generation of a luciferase reporter plasmid in which the target gene of interest is cloned as the 3′ untranslated region (UTR) of *Renilla* luciferase; (2) co-transfection of miRNA shuttles with this reporter into any available cell line; and (3) a dual luciferase assay kit (Promega). In the following paragraph, we describe the general principles underlying this method.

First, we created a new dual luciferase vector called pSI-CHECK-DEST, which we modified from the commercial pSICHECK2 vector (Promega). Our new construct contains Gateway® destination vector sequences (DEST®; Invitrogen) that allow for rapid recombination of Gateway ENTR® (Invitrogen) vectors containing target genes of interest (Fig. 5). These gene-specific reporters are generated rapidly using LR Clonase enzyme (Invitrogen). The end result is a reporter plasmid (pSICHECK-GOI) expressing a fusion transcript in which the target gene of interest is inserted downstream of the *Renilla* luciferase stop codon, effectively making it the *Renilla* luciferase 3′ untranslated region (3′ UTR; Fig. 5). From this fusion transcript, only the *Renilla* luciferase coding region is translated into protein (Fig. 6). An artificial miRNA that silences the target gene of interest will also reduce *Renilla* luciferase protein expression (Fig. 6). Importantly, pSICHECK-GOI also contains a separate HSV-TK promoter-driven firefly luciferase gene, which is unaffected by the artificial miRNA and, therefore, serves as a transfection control (Fig. 6). Gene silencing can then be determined by measuring *Renilla* and firefly luciferase activity in lysates from transfected cells using a dual luciferase assay kit (Promega).

7.1. Generating the Luciferase Reporter Plasmid

The pSICHECK-DEST vector allows rapid recombination of Gateway ENTR vectors containing target gene sequences into the 3′ untranslated (3′ UTR) portion of the *Renilla* luciferase gene. Importantly, many Gateway®-ready cDNAs are commercially available (e.g., Open Biosystems, Origene, Addgene, and PlasmID). If an ENTR vector containing your gene of interest is not available through a commercial resource, one can be generated using standard PCR techniques and TOPO® cloning kits (pENTR/D-TOPO; Invitrogen). To create a pSICHECK vector containing your gene of interest, mix 150 ng of pSICHECK-DEST with 150 ng of pENTR-GOI and perform an LR reaction, following the manufacturer's instructions (LR Clonase; Invitrogen). There are several layers of selection to ensure efficient recombination. First, a successful LR reaction will replace the ccdB and chloramphenicol resistance genes in pSICHECK-DEST with the gene of interest. The bacterial ccdB gene is toxic to typical *E. coli* used in competent cells (e.g., TOP10 and DH5α) and, if expressed, bacterial colonies cannot grow. Additionally, pSICHECK-DEST also expresses the ampicillin (Amp) resistance

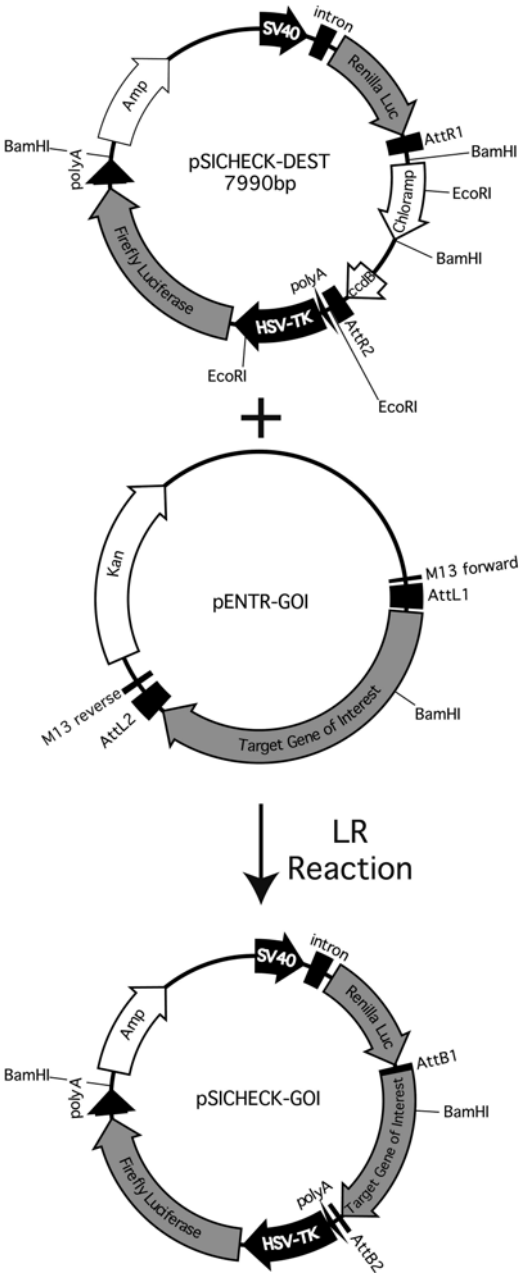

Fig. 5. Generating a luciferase reporter plasmid with the Gateway® system. pSICHECK-DEST is a new dual luciferase vector we created to facilitate cloning of the luciferase gene of interest fusion transcripts. Details are described in the text. Abbreviations: *SV40* SV40 promoter, *Luc* luciferase, *Chloramp* chloramphenicol resistance gene, *HSV-TK* herpes simplex virus thymidine kinase promoter, *Kan* kanamycin resistance gene, *Amp* ampicillin resistance gene, *GOI* gene of interest.

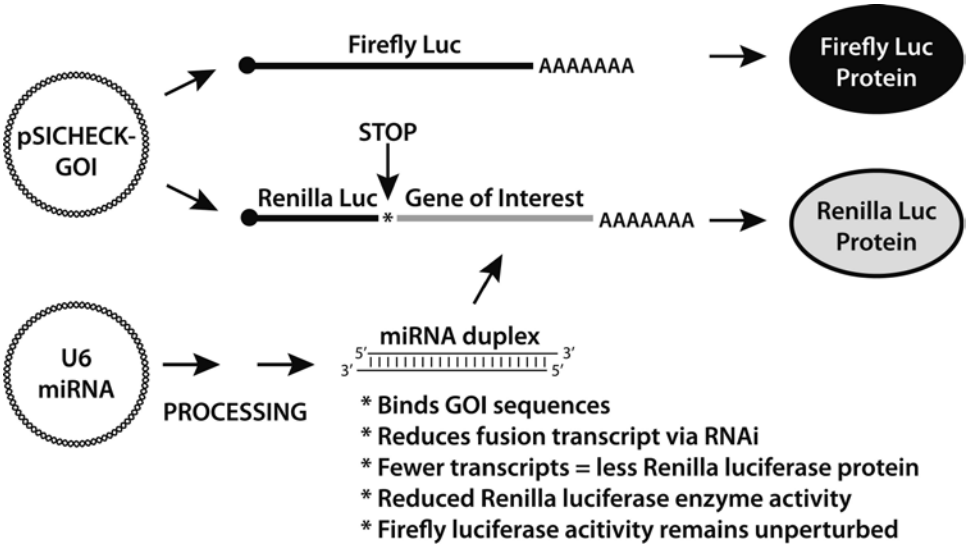

Fig. 6. Overview of luciferase assay screen for testing miRNA gene silencing. The pSICHECK-GOI plasmid, once constructed as described, expresses two different luciferase reporter genes. The first is a firefly luciferase reporter used to normalize transfections, and the second is a hybrid transcript in which the *Renilla* luciferase mRNA is fused with the target gene of interest (GOI). A STOP codon is placed between the *Renilla* coding region and the GOI sequence, so that a fusion mRNA is produced, but not a fusion protein. The GOI sequences are thus analogous to the 3′ UTR region of *Renilla* luciferase. Any inhibitory RNA (e.g. one arising from the U6 miRNA plasmid depicted here) that effectively silences the target GOI will therefore suppress *Renilla* luciferase, which can then be quantified using commercially available kits. Details are described in the text.

gene, while typical ENTR vectors are kanamycin resistant, thereby preventing the growth of any unrecombined ENTR vector colonies. Any living, ampicillin resistant bacterial colonies arising from LR recombination are likely correct, but restriction digests using, for example, *Bam*HI (Fig. 5) should be performed on miniprep DNA to confirm correctness of the plasmids.

7.2. Dual Luciferase Assay to Determine Gene Silencing Efficiency of Artificial miRNA Shuttles

For in vitro miRNA screening assays, we use HEK293s because of their rapid growth and ease of transfection using liposome reagents (e.g., Lipofectamine-2000; Invitrogen). Moreover, HEK293s possess all miRNA biogenesis and gene silencing machinery and have been used extensively for RNAi studies. Seed 2.5×10^4 HEK293 cells 1 day prior to transfection on 96-well plates. Since the goal of the experiment is to identify the most potent miRNA shuttle at the lowest dose, we typically perform screens using two different ratios of miRNA to target vector. Controls can include empty U6T6 and/or a nontargeting miRNA shuttle. If your gene of interest is not *E. coli* LacZ, the example miLacZ described in this chapter could be used as a nontargeting control. Table 1 shows plasmid amounts used in a typical transfection for an miRNA efficacy screen.

Perform each transfection in triplicate wells. After 48 h, measure *Renilla* and firefly luciferase activities using a dual

Table 1
Example transfection worksheet for miRNA efficacy screen using pSICHECK-GOI

Target plasmid	miRNA plasmid		
pSICHECK-GOI (ng)	miLacZ control (ng)	miGOI (ng)	Empty U6T6 (ng)
5	0	0	195
5	25	0	170
5	195	0	0
5	0	25	170
5	0	195	0
0	0	0	200

luciferase reporter assay kit (Promega) following the manufacturer's instructions. Determine gene silencing by plotting the ratio of *Renilla* to firefly luciferase activity in triplicate samples and normalize to "empty U6T6" or "control miRNA" data. This method provides a quick and consistent assay to identify lead miRNA shuttle vectors targeting your gene of interest, which is its major advantage. Results from this reporter assay are consistently translatable to natural transcripts, but we still advise that silencing of the natural target be confirmed using a second method, such as western blotting or real-time PCR.

8. Summary

RNA interference is a powerful tool for investigating basic biological questions and developing nucleic acid therapeutics. Artificial miRNA shuttles can be engineered to suppress any gene of interest using designs that closely mimic natural miRNA structures. As such, miRNA shuttles are excellent systems for achieving long-term gene silencing in many different cell and animal models. For plasmid- or vector-based RNAi expression systems, miRNA shuttles are arguably the best available system.

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