

# MicroRNA Sponge Production Using PCR-Based Concatemerization of Short DNA Oligonucleotides

## Research Article

### Abstract

MicroRNAs (miRs) are small, non coding RNAs encoded within our genome that regulate gene expression by silencing messenger RNA (mRNA) transcripts. Since being discovered in humans in 2001, much has been learned concerning the many cellular activities that miRs can affect. MiRs have been implicated in almost every cellular activity, including cell fate determination, stress response, metabolism, apoptosis, and carcinogenesis. Importantly, alterations in miR activity due to mutation or mis expression have been repeatedly shown to result in tumorigenesis and disease. However, because of their relatively recent discovery, therapeutic tools to suppress miRs continue to be developed. One extremely promising way to inhibit miRs is to use miR sponges which consist of ~15 miR "target" sites that can specifically bind and inactivate particular miRs. Unfortunately, miR sponge production has proven to be problematic thus far as current production methods involve either costly commercial synthesis or low throughput ligation-based cloning. However, we have recently developed a novel, PCR-based method that allows sponge production in a rapid, high throughput manner. In all, PCR amplification, cloning, sequence confirmation and large scale production are readily achievable in less than two weeks and at a significantly lower cost than current methods.

**Keywords:** microRNA; miRNA; miR; miR inhibitors; miR sponge; PCR

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**Abbreviations:** bp: base pair; ceRNA: competing endogenous RNAs; circRNA: circular RNA; ciRS: circular RNA Sponges; lincRNA: long intervening noncoding RNAs; LNA: Locked Nucleic Acid; miR: microRNA; mRNA: messenger RNA; nt: nucleotide; PCR: Polymerase Chain Reaction; pre-miR: precursor microRNA; pri-miR: primary microRNA; RISC: RNA Induced Silencing Complex; siRNA: small interfering RNA; UTR: Untranslated Region

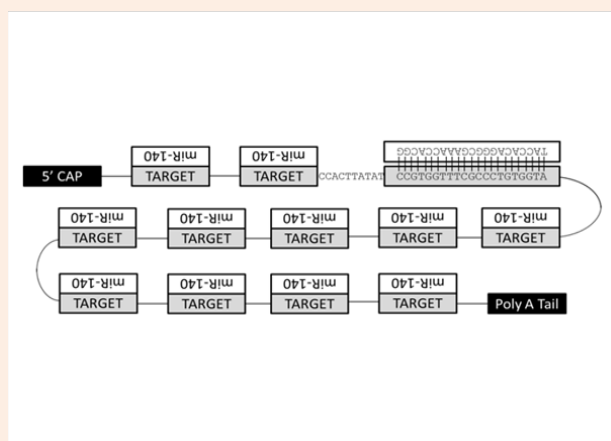
### Introduction

MicroRNAs (miRs) are small, ~20 nucleotide (nt) non-coding RNAs that act as regulators of gene networks. Since being discovered in *Caenorhabditis elegans* in 1993, studies have identified over 2,500 unique human miRs and over 35,000 mature miR products across species. MiRs are widespread in higher eukaryotes and similar in function to small interfering RNAs (siRNAs), in that they silence specific messenger RNAs (mRNAs). Generation of miR from endogenous hairpin-shaped transcripts occurs in two main stages: within the nucleus and without [1]. First, the miR gene is transcribed within the nucleus by either RNA Polymerase II or III producing a long primary molecules, called a pri-miR typically composed of several thousand nts [2]. The pri-miR is then cut by Drosha, yielding a ~70 nt long precursor stem loops (pre-miR). Next, the pre-miR hairpin is exported to the cytoplasm by exportin-5, where Dicer cuts it down to a ~22nt duplex. One strand is degraded and the other is retained as the mature miR that associates with the RNA induced silencing complex (RISC) [1,3]. From there, the complementarity between the 6-8 nt 'seed' region of the miR and the 'seed match' region on the mRNA determines the fate of the mRNA [4]. Importantly, miR binding does not have to be perfect in order to have an effect.

When a miR seed binds to the mRNA seed match, it can result in one of two things: mRNA cleavage in the event of perfect full-length miR complementarity, or translational repression if the match is imperfect. Importantly, a single miR can influence the activity of numerous mRNAs, an ability which in turn allows miRs to form intricate regulatory networks [5].

Altered activities of several miRs due to mutation or mis expression have now been shown to result in disease onset and/or tumorigenesis [6-9]. While miR mis regulation is implicated in numerous malignancies, therapeutic tools for miR suppression have only recently been developed [6]. Commercially available miR inhibitors fall into three major categories: antagomirs [10], synthesized sponges [11], or antisense inhibitors [12]. Antagomirs, better known as LNAs (locked nucleic acids) or anti-miRs, are chemically modified nucleic acids that strongly bind a target miR by traditional complementary base pairing. The thermodynamic stability of LNA binding keeps them tightly associated with their targets, but also allows for imperfect base pairing at high temperatures. Unfortunately, LNAs carry the risk of off-target effects [13] making them less desirable candidates for therapeutic endeavors despite their demonstrated utility as a tool for basic research [11]. In contrast, antisense inhibitors (such as Ambion's mirVana) contain an unmodified RNA strand (which binds targets with normal affinity) and a chemically modified strand that cannot associate with RISC. Although this class of inhibitors is not hindered by the potential toxicity associated with LNAs, these antisense inhibitors can still prove problematic for many researchers in terms of the cost associated with their production [12]. Significantly different in design than either of the synthetic inhibitors, the third type of miR inhibitor currently

employed is the miR sponge. miR sponges are RNA transcripts with repeated binding sites forming a concatemer of targets for a specific miR (Figure 1). Sponges can be constructed with repeats of any sequence, but sponges with central mismatches, called a central bulge, have been shown to be more effective at inhibiting miR function than perfectly complementary sponges [11]. To date, sponges have been synthesized by either commercial synthesis or T4 ligation reactions. While more cost effective than commercial synthesis, due to the linear nature of their reaction mechanism, T4 ligation reactions are notoriously low throughput and typically require large amounts of starting material to produce relatively low product yields [14].



**Figure 1:** MicroRNA target binding: Cartoon illustrating a miR-140 miRNA sponge. Expressed from a eukaryotic promoter, sponges consist of capped, polyadenylated mRNAs bearing multiple miRNA “Target” sites. In all, twelve target sites are illustrated base paired to complementary miR-140 miRNAs.

Excitingly, we have developed an entirely novel, rapid, PCR-based methodology that greatly facilitates sponge production. The concatemerizing PCR technique we present here represents a cost-effective and practical method for miR sponge production. Instead of time-consuming ligation reactions that produce little overall yield, concatemerizing PCR utilizes amplification steps and exponential growth to quickly generate large quantities of sponge amplicons that consist of an easily user-defined number of miR binding sites. Importantly, as prominent roles for miRs in disease development and progression continue to be described [7-9,15] knocking down miRs in the laboratory and clinic has become an increasingly significant endeavor, and the method of sponge production described here constitutes an innovative, inexpensive, high yield means of miR inhibition.

## Materials and Methods

### Primer design

Approximately 40 nucleotide forward primers were constructed with GGTGAATATA at both the 5' and 3' ends flanking an internal sequence to be repeated and concatemerized. Reverse primers were ordered exactly as the reverse complement of the forward primer allowing concatemerization. Each sponge was concatemerized with the following primers:

**miR140 sponge:** GGTGAATATATACCACAGGGCGAAACCACGGG-GTGAATATA,

TATATTCACCCCGTGGTTTCGCCCTGTGGTATATATTCACC

**SPG1:** GGTGAATATATTAATACGTGACCTATTAATGGTGAATATA, TATATTCACCATTAATAGGTCACGTATTAATATATTCACC

**SPG2:** GGTGAATATATAGCTTATCAGACTGATGTTGAGGTGAATATA, TATATTCACCTCAACATCAGTCTGATAAGCTATATATTCACC

**SPG3:** GGTGAATATACTAATACTGCCTGGTAATGATGAGGTGAATATA, TATATTCACCTCATCATTACCAGGCAGTATTAGTATATTCACC

**SPG4:** GGTGAATATACTAATACTGAAAGGTAATGATGAGGTGAATATA, TATATTCACCTCATCATTACCTTTCAGTATTAGTATATTCACC

**SPG5:** GGTGAATATATGGGAGGGGAGAGGCAGCAAGCAGGTGAATATA, TATATTCACCTGCTTGCTGCCTCTCCCTCCCATATATTCACC

**SPG6:** GGTGAATATATGGGAGGGGACGGCAGCAAGCAGGTGAATATA, TATATTCACCTGCTTGCTGCCTGCCCTCCCATATATTCACC

**SPG7:** GGTGAATATACATGCTGACCTCCCTCTGCCCCAGGGTGAATATA, TATATTCACCTGGGGCAGGAGGGAGGTCAGCATGTATATTCACC

**SPG8:** GGTGAATATACATGCTGACCAAACCTCTGCCCCAGGGTGAATATA, TATATTCACCTGGGGCAGGAGTTTGGTCAGCATGTATATTCACC

### Concatemerizing PCR

PCR amplifications were performed in 50  $\mu$ L reactions at standard concentrations [10  $\mu$ L of Bioline 5xMyTaq buffer (comprised of 5.0 mM dNTPs, 15 mM MgCl<sub>2</sub>), 1.0  $\mu$ L Taq (Bioline, USA, Inc., Randolph, MA), 0.2  $\mu$ M of each primer]. The following cycling parameters: 94°C – 2:00 min, (94°C- 30 s 45°C - 10 s, 72°C - 30 s)  $\times$  2 cycles, (94°C - 30 s, 40°C - 30 s, 72°C - 30 s)  $\times$  29 cycles, 72°C-1:00 minute, 4°C-or ever, were utilized. Following this, resulting amplicons were separated on a 1% agarose gel and desired sponge length was extracted. See “Supporting Information - Supplemental Protocol” for additional details.

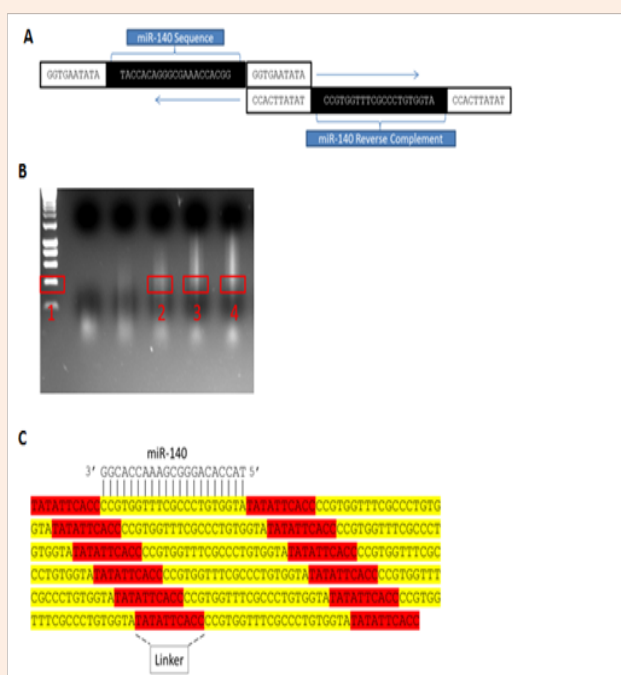
### Vector cloning

The band corresponding to desired sponge length was excised from the appropriate lane at ~400 bp. Gel extractions were cloned into a pcr2.1-TOPOTA vector (Invitrogen). Concatemers were next excised from pcr2.1-TOPOTA and cloned into a mammalian expression vector (PCI, Promega, Madison, WI) using EcoR1 restriction sites which placed the sponge into the 3'UTR of the T7 promoter. Sponge orientation in PCI vector was determined by commercial sequencing (Functional Biosciences, Madison, WI). Sequencing primers for final constructs were: PCIF, TAATACGACTCACTATAGG; PCIR, AATAGCATCACAAATTCAC.

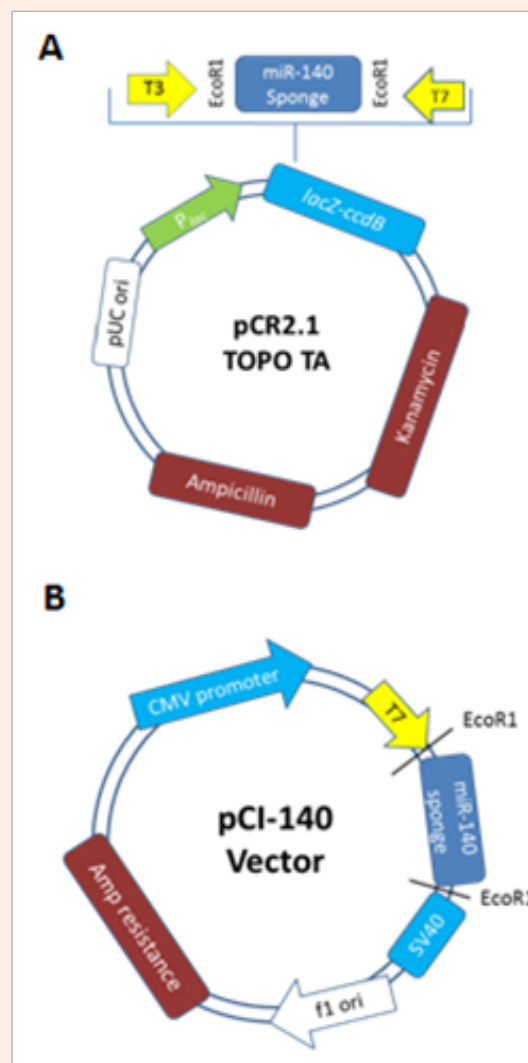
## Results and Discussion

To achieve sponge production, ~40 nt forward primers consisting of a simple linker (GGTGAATATA) flanking the 5' and 3' ends of an internal ~20 nt miR sequence to be repeated across the sponge were designed and synthesized commercially. Reverse primers (the exact reverse complement of the forward primer) were also ordered, thereby allowing concatemerization of the sequence complementary to the miR by the mechanism depicted in (Figure 2A). PCR amplifications were initially performed in 50  $\mu$ L reactions at standard concentrations. Following amplification, PCR products were separated by gel electrophoresis, and regions corresponding to ~10 repeats (~400 bp) were manually excised (Figure 2B) then cloned into the PCR 2.1-TOPOTA (Invitrogen) vector (Figures 2C & 3A). Next, to allow eukaryotic expression,

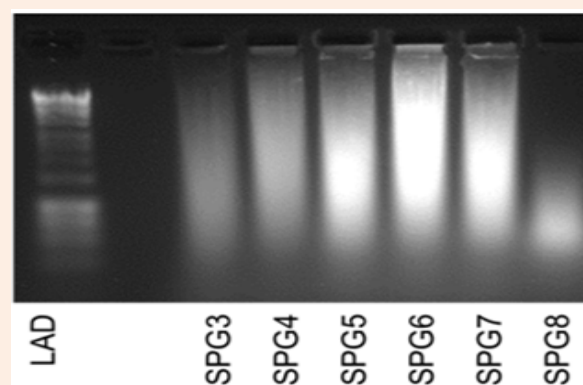
sponges were removed from TOPO by the restriction digest of EcoRI sites immediately flanking both sides of the TOPO insertion, after which they were non-directionally sub cloned into a mammalian expression vector (PCI, Promega) linearized with EcoRI (Figure 3B). Multiple colonies were isolated from resultant transformations and commercially sequenced (Functional Biosciences, Madison, WI). While expression vector subcloning could alternatively be performed in a unidirectional manner following determination of orientation in PCR 2.1-TOPOTA by sequencing, this strategy allowed the simultaneous generation of both an expression vector producing a desired sponge and an identical expression vector producing the reverse complement of the sponge-an ideal transfection control. Importantly, Figure 2 demonstrates how a single round of PCR can be successfully employed to concatemerize single oligonucleotide complements into markedly larger molecules tens of thousands of base pairs in length, from which any desired number of repeated miR target sites can be selected and utilized. Finally, we tested the versatility of our methodology by designing and attempting to amplify eight additional, unique sponges. Markedly, we found all eight primer sets robustly concatemerized under the conditions outlined here (Figure 4).



**Figure 2:** MicroRNA sponge production by concatemerizing PCR: **A.** Complementary binding and elongation from concatemerizing primer linker sequences during PCR amplification. miR Seq R/C, reverse complement of mature microRNA. **B.** Agarose gel separation of the products of three distinct concatemerizing PCRs. Box 1, Hyperladder I. Boxes 2-4, the regions (~400 bp) excised for sponge cloning. Distinct concatemerizations performed with the primers listed in Methods - Primer design. **C.** MiR-140 sponge expression vector sequence confirming the presence of eleven miR-140 target sites (yellow) separated by linkers (red). The sequence is shown as it would be found in the expressed mRNA with putative miR-140 miRNA binding indicated.



**Figure 3:** MiR-140 sponge clones. **A.** PCR4-TOPO TA plasmid containing miR-140 sponge between two EcoRI sites. **B.** PCI mammalian expression vector containing miR-140 sponge.



**Figure 4:** Additional concatemerizing PCRs: Agarose gel separation of the products of six distinct concatemerizing PCRs as in **Figure 2B**: Concatemerizations were performed with the primers listed in Methods - Primer design.



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>hsa-miR-140-3p MIMAT0004597
Original: TACCACAGGGTAGAACCACGG
Altered: TACCACAGGGCGAAACCACGG
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**Figure 5:** MiR-140 primer design: Original mature miR-140 sequence with seed in red. Altered miR-140 sequence with central mismatch ("bulge") indicated by highlight.

## Conclusion

In summary, as miRs clearly play prominent roles in disease, development and normal cellular metabolism [15], knocking down miRs has become an important area of research. As such, the discovery of several types of naturally occurring miR sponges strongly supports the application of engineered miR sponges in miR inhibition. Naturally expressed circ RNA or circular RNA sponges (ciRS) can contain up to 70 miR mismatched target sites capable of binding multiple miR families [16] much like newly discovered (ceRNAs) which also demonstrate endogenous miR sponge-like activity [17]. CeRNAs allow non-coding RNAs, such as miRs, to communicate via competition for mRNAs, with ce RNA function depending on miR concentration. CeRNAs contain binding sites for various miRs allowing them to regulate multiple miR families [18]. In addition, several other classes of non-coding RNAs may also function as endogenous miR sponges (e.g. 3' UTR RNAs and linc RNAs) [19].

Currently, sponges are synthesized by either commercial synthesis or T4 ligation reactions. While more cost effective than commercial synthesis, due to the linear nature of their reaction mechanism, T4 ligation reactions are notoriously low throughput and typically require large amounts of starting material to produce relatively low product yields [14]. In contrast, the method of sponge production we describe here represents a highly efficient, enzymatic reaction utilizing Taq polymerase to readily generate potent miR inhibitors that mimic endogenous sponges in a shorter time frame and at a substantially lower cost than existing production methods. Importantly, sponges can be constructed with repeats of any short sequence and designed to be perfect matches to miRs or instead to contain mismatches, or a central bulge (Figure 5), which has been suggested to be more effective at inhibiting miR function than perfectly complementary sponges [11]. In addition, while we developed this PCR methodology to concatemerize miR target sites, we suggest this strategy could also be successfully utilized for other research purposes such as the concatemerization of transcription factor recruitment sites in expression constructs.

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