MicroRNA sponge production using PCR-based concatamerization of short DNA oligonucleotides

Abstract
Micro RNAs (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 tumorogenesis 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

Abbreviations:
Bp, base pair; CE RNA, competing endogenous RNA; circRNA, circular RNA; cholR, circular RNA sponge; LNA, locked nucleic acid; miR, microRNA; mRNA, messenger RNA; MT, 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
Micro RNAs (miRs) are small, ~20 nucleotide (nt) non-coding RNAs that act as regulators of gene networks. Since being discovered in C. elegans in 1993, studies have identified over 2,500 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. First, the miR gene is transcribed within the nucleus by either RNA Polymerase II or III producing a long primary molecule, called a pri-miR typically composed of several thousand nts. The pri-miR is then cut by Drosophila, yielding a ~70nt 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). From there, the complementarity between the 6-8nt ‘seed’ region of the miR and the ‘seed match’ region on the mRNA determines the fate of the mRNA. 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.

Altered activities of several miRs due to mutation or mis-expression have now been shown to result in disease onset and/or tumorogenesis. While miR mis regulation is implicated in numerous malignancies, therapeutic tools for miR suppression have only recently been developed. Commercially available miR inhibitors fall into three major categories: antagonomers, synthesized sponges, or antisense inhibitors. Antagonomers, better known as LNA (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 making them less desirable candidates for therapeutic endeavors despite their demonstrated utility as a tool for basic research. 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. Significantly different in design than either of the synthetic inhibitors, the third type of miR inhibitor currently employed is the miR sponge. Sponges are RNA transcripts with repeated binding sites forming a concatamer 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. To date, sponges have been synthesized by either commercial synthesis or T4 ligation reactions. While more cost effective than commercial synthesis, due...
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Excitingly, we have developed an entirely novel, rapid, PCR-based methodology that greatly facilitates sponge production. The concatemerizing PCR technique we present here accomplishes 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,13-15 knocking down miR levels in the laboratory and clinic has become an increasingly significant endeavor, and the method of sponge production described here constitutes an innovative, 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 sequence to be repeated across the sponge (Figure 1). The following primers were designed and synthesized commercially. Reverse primers with GGTGAATATA at both the 5’ and 3’ ends flanking an internal ~20nt 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. Primer amplifications were successfully performed in 50μL reactions at standard concentrations. Following amplification, PCR products were separated by gel electrophoresis, and regions corresponding to ~10 repeats (~400bp) were manually excised and cloned into the plasmid vector for sequencing.

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 ~20nt 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. Primer amplifications were successfully performed in 50μL reactions at standard concentrations. Following amplification, PCR products were separated by gel electrophoresis, and regions corresponding to ~10 repeats (~400bp) were manually excised and cloned into the plasmid vector for sequencing.

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-TOPO vector (Invitrogen). Concatemers were next excised from pcr2.1-TOPO and cloned into a mammalian expression vector (PCI, Promega, Madison, WI) using EcoRI 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, AATAGCATCACAAATTTCAC.

SPG8: GGTGAATATATCGTGACCAATCTCCGCCCCAGGTTGGAATATA, TATATCCACCTGGGACAGAGGGTGTGCAGCATGATATTCACC

Concatemerizing PCR

PCR amplifications were performed in 50μL reactions at standard concentrations [10μL of Bioline 5xMyTaq buffer (comprised of 5.0μM dNTPs, 15mM MgCl2), 1.0μL Taq (Bioline, USA, Inc., Randolph, MA), 0.2μm of each primer].The following cycling parameters: 94°C–2:00min, (94°C-30s 45°C-10s, 72°C-30 s)×2cycles, (94°C-30s, 72°C-30s)×10cycles, 72°C-1:00min, 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.

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

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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 micro RNA.
B. Agarose gel separation of the products of three distinct concatemerizing PCRs. Box 1, Hyper ladder I. Boxes 2-4, the regions (~400bp) 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 mi RNA binding indicated.

**Figure 3** MiR-140 sponge clones.
A. PCR4-TOPO TA plasmid containing miR-140 sponge between two EcoR1 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.

**Conclusion**

In summary, as miRs clearly play prominent roles in disease, development and normal cellular metabolism, 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 much like newly discovered (ce RNAs) which also demonstrate endogenous miR sponge-like activity. Ce RNAs allow non-coding RNAs, such as miR s, to communicate via competition for miRNAs, with ce RNA function depending on miR concentration. Ce RNAs contain binding sites for various miRs allowing them to regulate multiple miR families. In addition, several other classes of non-coding RNAs may also function as endogenous miR sponges (e.g. 3’ UTR RNAs and linc RNAs).

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. 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 miR Rs 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. 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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Conflict of interest

The author declares no conflict of interest.

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