Published in final edited form as:

Recent Adv DNA Gene Seq. 2014 ; 8(1): 2–9.

Methods and Compositions for Amplification and Detection of microRNAs (miRNAs) and Noncoding RNAs (ncRNAs) Using the Signature Sequence Amplification Method (SSAM)

Stephen D. Ginsberg^{1,2,3,*} and Shaoli Che^{1,2}

¹Center for Dementia Research, Nathan Kline Institute, Orangeburg, NY, USA

²Department of Psychiatry, New York University Langone Medical Center, New York, NY, USA

³Department of Physiology & Neuroscience, New York University Langone Medical Center, New York, NY, USA

Abstract

The signature sequence amplification method (SSAM) described herein is an approach for amplifying noncoding RNA (ncRNA), microRNA (miRNA), and small polynucleotide sequences. A key point of the SSAM technology is the generation of signature sequences. The signature sequences include target sequences (miRNA, ncRNA, and/or any small polynucleotide sequence) flanked by two DNA fragments. Target sequences can be amplified through DNA synthesis, RNA synthesis, or the combination of DNA and RNA synthesis. The amplification of signature sequences provides an efficient and reproducible mechanism to determine the presence or absence of the target miRNAs/ncRNAs, to analyze the quantities of the miRNAs in biological samples, and for miRNA/ncRNA profiling.

Keywords

Amplification kit; first oligonucleotide; gene expression; gene targets; *in vitro* transcription; microarray; microRNAs; molecular biology; neuroscience; noncoding RNAs; novel gene identification; polynucleotide sequences; qPCR; RNA amplification; RNA; second oligonucleotide; signature sequence; streptavidin beads

1. INTRODUCTION

The present invention, termed the signature sequence amplification method (SSAM) is designed for the amplification of small polynucleotides, such as microRNAs (miRNAs), small interfering RNAs (siRNAs), short hairpin RNAs (shRNAs), longer species such as long non-coding RNAs (lncRNAs), and other noncoding RNAs (ncRNAs). The central

© 2014 Bentham Science Publishers

*Address correspondence to this author at the Center for Dementia Research, Nathan Kline Institute, NYU Langone Medical Center, 140 Old Orangeburg Road, Orangeburg, NY 10962, 845-398-2170, USA; Tel:/Fax: 845-398-5422; ginsberg@nki.rfmh.org. Send Orders for Reprints to reprints@benthamscience.net

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

dogma of molecular biology and genetics is that within a cell DNA is transcribed into messenger RNA (mRNA) and mRNA is subsequently translated into a corresponding protein [1, 2]. As with many general biological principles, this view appears to be generally valid. Accumulating evidence, however, including sequencing of the human genome and the continued sequencing of relevant animal models has demonstrated that there appears to be more information in the genome in terms of protein synthesis control than previously realized [3–5]. Multiple lines of research demonstrate that ncRNAs may play a central role in translational regulation. For example, <1%–3% of mRNAs are translated into proteins [2, 6]. Importantly, miRNAs, are a class of short 17–25-nucleotide (nt) RNA molecules have been shown to have critical functions in a wide variety of biological processes [7, 8]. miRNAs are synthesized from larger miRNA transcripts that fold to produce hairpin structures and serve as substrates for an enzyme of the RNase III family termed cytoplasmic Dicer [9, 10]. They are endogenous ncRNAs, which can regulate gene expression of multiple mRNAs [11, 12]. Since the identification of miRNAs in 1993 [13], a growing list of additional miRNAs has been expanding steadily. To date, ~1870 miRNAs have been identified in human (<http://www.mirbase.org>; release 20) [14, 15], and each of these miRNAs has been posited to interact with 5–10 mRNAs [16]. It is estimated that ~60–70% of all expressed genes are regulated by miRNAs [17, 18]. With the exception of certain viruses, most miRNAs reduce gene expression through reduction of the abundance and/or the translational efficiency of target mRNAs [19].

Studies have shown that miRNAs can be expressed in a tissue-specific or cell-specific manner, including brain regions relevant for neurodevelopmental, neuropsychiatric, and neurodegenerative disorders [20–27] as well as at the level of the dendrite and the synapse [26, 28–31]. Essentially, a mosaic of miRNA expression levels regulates cell growth, differentiation, and other critical parameters throughout various cell types and tissues [32, 33]. miRNA expression levels are controlled tightly during development and are critical for generalized homeostasis [34, 35]. Furthermore, many pathological conditions appear to be related to dysfunction of miRNA expression, including several types of cancer [36–38].

miRNA expression profiling is an ideal method to study expression levels and regulation of miRNAs, similar to current paradigms examining mRNA expression levels at the global, regional, and cellular level [39–41]. However, miRNA profiling poses more challenges than mRNA profiling. One major obstacle when profiling miRNAs is the low expression level of ncRNAs, including miRNAs. As a result, large quantities of input RNA are required for miRNA profiling. This requirement makes it difficult to study tissue-specific and cell-type specific expression of miRNAs. Another difficulty of profiling miRNAs is their small size, as they are predominantly 22–25 nt [7, 8, 13]. The small size of miRNAs makes direct amplification difficult with conventional RNA amplification and/or qPCR-based strategies, although several groups have performed modification procedures of RNA amplification methods to identify individual miRNAs [42–46]. Unlike mRNAs, miRNAs lack an appropriate sequence to anchor a primer for the first strand DNA synthesis, which is typically the first step of the majority of RNA amplification procedures [47, 48]. Moreover, the small size of miRNAs makes them difficult to hybridize to array platforms, because of the unstable hybridization of short oligonucleotides to the complementary sequences. Unfortunately, positive signals generated by short oligonucleotides are generally weak,

which reduces the overall detection sensitivity of the assay, and high background is typically a problem due to high concentrations of probe and longer exposure times required for signal detection.

Indirect methods are currently employed to amplify miRNAs. In general, these approaches attempt to attach a known sequence to target miRNAs through ligation or terminal transferase, which is followed by amplification steps through either DNA or RNA synthesis. These strategies may increase the signal strength but do not substantially increase sensitivity, because they invariably increase the background nonspecific hybridization. Another tactic is to employ direct labeling of total RNA (presumably including miRNAs) followed by hybridization to immobilized probes. Direct labeling methods suffer from low sensitivity and specificity, and typically require starting material >10–20 µg. To increase the sensitivity and specificity, small RNAs can be enriched *via* acrylamide gel electrophoresis. The enrichment of small RNAs increases the specificity, but not the sensitivity of miRNA profiling due to the low yield of miRNAs. This method is suitable for Northern blot analysis of individual miRNAs, but is not generally sensitive enough for profiling of multiple miRNAs from small input sources. Another method to amplify miRNA employs real-time quantitative PCR (qPCR). This method has been demonstrated to be successful for individual miRNAs when sample RNA is abundant [42, 49, 50]. However, qPCR miRNA profiling is difficult to ‘scale up’ for minute quantities of input RNA species, since each miRNA has to be amplified and detected separately. Moreover, the method is difficult to perform with multiple samples and therefore suffers low throughput as compared to medium- and high-throughput array methods. Thus, there is a need for improved miRNA amplification methods that overcome drawbacks in existing methods.

2. SSAM APPROACH

A principal objective and advantage of the present invention is to provide a reliable and reproducible miRNA amplification method for investigations using small input samples in many diverse disciplines. The SSAM approach is also a method for the amplification of small polynucleotides encompassing any and all ncRNAs. Importantly, the SSAM technology does not skew the original quantitative relationships, and can be used for high-throughput methods. The SSAM approach provides methods for amplifying ribo- and deoxyribo- small polynucleotides by the creation of a pool of signature sequences to the target molecules. Through the generation of signature sequences, the target miRNA species can be amplified *via* DNA synthesis, RNA synthesis, or the combination of DNA and RNA synthesis (Fig. 1). The amplification of signature sequences provides an efficient and reproducible means to determine presence or absence of the target miRNAs, analyze the quantities of the target miRNAs, and miRNA profiling. With a slight modification of the first oligonucleotides (FONs) and second oligonucleotides (SONs) required for the procedure, SSAM technology can also be used to screen for unknown ncRNAs including novel miRNAs. The magnitude of miRNA amplification through the generation of signature sequences can be readily increased by several means; including multiple rounds of amplification; the combination of limited cycle PCR and *in vitro* transcription (IVT). Detection sensitivity can be increased by hybridization stability, such as increasing the length of final amplification products, including employing one or several nucleotide

analogs, such as locked nucleic acids in probes [51, 52], to which the amplified miRNAs hybridize. Alternatively, a labeled short oligonucleotide sequence can be attached to amplified miRNA, which increases signal strength and detection sensitivity.

2.1. FONs and SONs

A unique feature of the present invention is the stepwise construction of signature sequences. This functionality distinguishes the present technology from all the other methods available currently, where typically all of the oligonucleotide primers required for the detection scheme are added the reaction mixture simultaneously. A signature sequence is composed of two distinct oligonucleotide primers (FON and SON) and a miRNA serves as a primer for the first step DNA synthesis (Figs. 1–3). The experimental design ensures that FON and SON primers only interact with each other in a sample preparation when target miRNAs are present. The generation of a signature sequence population to the desired target ncRNA/miRNAs/small polynucleotide sequences in the SSAM technology is fundamentally different from previous methods which attach a variety of sequences of interest, such as a bacteriophage RNA synthesis promoter, to a cDNA copy of mRNA through reverse transcription [48, 53]. Signature sequences are generated in the presence of target miRNA. However, the ‘first strand DNA’ in the present invention is not a cDNA copy of a RNA molecule. Rather, it is a DNA copy of a DNA molecule. Instead of being a template, miRNA serves as a primer, and a sequence-specific FON is a template for a DNA dependent DNA synthesis (Figs. 3, 4). Although a signature sequence includes a target miRNA sequences, a signature sequence is designed to be significantly longer than the target miRNA sequence.

FONs and SONs are designed on the basis of confirmed mature miRNA sequences so that they will only amplify and detect mature, but not immature, pro- or pre- miRNAs when total RNA populations are used as starting samples. Since only mature miRNAs are presumed to be biologically functional, the present invention has the advantage over many commercially available direct labeling methods in distinguishing functional from nonfunctional miRNAs. For the same reason, the present approach eliminated the need to enrich or purify miRNAs prior to miRNA detection. Quantification can be performed through direct assessment of sequence abundance or through quantification of label incorporation (fluorescent, radioactive, and biotin-conjugated probes can be employed) similar to our protocols designed for mRNA amplification, notably terminal continuation (TC) RNA amplification [41, 48, 54].

The present invention may also be used to screen for unknown miRNAs/small polynucleotide sequences. Instead of attaching a known sequence to the 3' of FONs, a random sequence can be attached. The 5' of FONs will have the requisite functional sequences for the downstream processing of DNAs. After the first DNA synthesis step, and clearing the samples of free unbound FONs, the SONs will be added into the sample. The 3' of the SONs will also be comprised of a random sequence and the 5' will contain necessary functional sequences for DNA amplification and cloning. The double stranded DNAs can either be cloned directly or following an amplification step. The clones can be selected by appropriate antibiotic resistance and confirmed by direct sequencing.

3. MATERIALS AND METHODS

3.1. Definition of Terms

A “target ncRNA”, “target miRNA”, or a “potential target miRNA” is a short ribopolynucleotide sequence of interest, for which amplification is desired. The sequences of the target ncRNA and/or miRNA are either known, or can be unknown.

The essential feature of a signature sequence is that it is a unique representative of a target ncRNA and/or miRNA. A signature sequence is formed by connecting appropriate FONs and SONs in the presence of target ncRNAs and/or miRNAs. The presence and quantity of a signature sequence directly reflects the presence and quantity of the target miRNA in the sample. A signature sequence is designed such that it has all the necessary regions for the downstream molecular manipulations, such as PCR, IVT, qPCR, and/or enzymatic modification. A signature sequence or a FON or a SON may also be attached with a label, such as a biotin molecule, to facilitate its detection or the clearing of excess FON and SON (Fig. 3).

3.2. SSAM Outline

The key steps of the SSAM approach consists of (a) hybridizing a target miRNA/small polynucleotide sequence to a FON (incubating a biological sample with a desired pool of FONs to hybridize a pool of target miRNAs in the desired sample); (b) extending the target miRNA/small polynucleotide sequence hybridized to a FON (including multiple miRNAs hybridized to the desired FONs) with a DNA polymerase to generate a (pool of) RNA/DNA double-stranded hybrid molecule(s); (c) cleaving the target miRNA/small polynucleotide sequence in the double stranded DNA/RNA hybrid molecules of step (b) which generates a double stranded DNA molecule with 3' overhangs; (d) incubating the sample generated at step (c) a pool of the SONs. The SONs have 3' sequences complimentary to the 3' overhangs of the double stranded DNA molecules generated in step (c) (Fig. 1). (d) Steps (a) to (d) are followed by alternative steps (e1) or (e2). Step (e1) involves ligating the SON with a DNA ligase to the DNA fragments of the double-stranded DNA molecule of step (d), thereby generating a pool of partially double stranded signature sequences to the target miRNAs. Step (e2) involves annealing SONs and fill in the overhang of FONs with a DNA-dependent DNA polymerase to generate a pool of double stranded signature sequences (Fig. 1).

3.3. First Strand DNA Synthesis

For the first step DNA synthesis, the FON and/or FONs are added into a sample containing (or potentially containing) at least one target miRNA/small polynucleotide sequence. The FONs and target miRNAs/small polynucleotide sequences will anneal to each other at their 3' ends with extruding 5' ends of the FONs. The target miRNAs that anneal to appropriate FONs will then act as DNA synthesis primers, whereas the extruding 5' end of a FONs will act as DNA synthesis templates. In the presence of a proper DNA polymerase and other component for DNA synthesis, double stranded DNA/RNA hybrids are formed.

Alternatively, the 3' end of FON may contain a DNA sequence complementary to a partial miRNA sequence. In the presence of a proper DNA polymerase, such as reverse

transcriptase, the FON can be further elongated to the full-length of the target miRNA. This method will increase specificity of the reaction, because a SON can be designed to contain only part of the target miRNA sequence synthesized in the previous step.

3.4. Digestion of DNA/RNA Hybrid Molecules

After the first DNA synthesis reaction, small target ribopolynucleotide sequences formed in DNA/RNA hybrid molecules are digested with an appropriate RNA endonuclease, such as RNase H. A pool of SONs is then added to the sample. The DNA copy of at least a partial target miRNA sequence is necessary in a SON, through which a SON anneals to a corresponding FON. In the presence of a DNA ligase, a SON annealed to a corresponding FON ligates to the DNA fragment generated in the first strand DNA synthesis and remains after the enzymatic digestion of the target miRNA. Alternatively, the 3' end overhangs of a FON and a SON annealed together can be filled by a DNA polymerase using annealed both 3' end as primers.

Clearing residual FONs in the sample before adding SONs are often desirable and may be considered essential. FONs can be removed by any methods described as following. (a) enzymatically digesting the single stranded free FONs with exonuclease I, which digest single stranded DNAs, whereas double stranded RNA/DNA hybrid molecules are protected from digestion, and can be processed for downstream amplifications (Fig 2). (b) The use of a restriction nuclease digestion site and a binding molecule such as biotin are included at the 5' end of FONs. In the absence of a target small RNA, the complementary strand or the displacement strand will not be synthesized (Fig. 3). In the situation of (a) a restriction endonuclease such as BMSI will digest the biotin moiety (Fig. 4) and a newly synthesized DNA displaces the biotin labeled strand. In both situations, excess FONs can be cleared from the reaction by streptavidin (SAv) conjugated beads (Figs. 3, 4).

3.5. Processing of SONs

SONs are added into the reaction mixture after all the free FONs are cleared. SONs hybridize to the 3' end overhang after the digestion of miRNA in the molecule. The signature sequence is generated by either ligation or a DNA synthesis reaction. As with the clearance of FONs, the samples can also be cleared of the SONs before proceeding to downstream amplification and subsequent functional genomics applications.

4. RESULTS: AMPLIFICATION OF mir-9

The SSAM methodology is demonstrated using mir-9, a miRNA species present in brain. Our group has similar results with brain-enriched mir-374 in mouse and postmortem human brains as well as alternatively spliced tau isoforms (including both 3-repeat tau and 4-repeat tau isoforms) in postmortem Alzheimer's disease and related tauopathies [55, 56]. Total RNA is isolated from mouse hippocampus for mir-9 detection. The process of SSAM detection consists of five steps: 1) synthesis of the complementary strand of the FON using the target miRNA (mir-9) as primer; 2) digestion of residual FON with exonuclease I 3) RNase digestion of mir-9 and attachment of a SON to the FON; 4) amplification of the "signature sequence" of the target miRNA (Figs. 4, 5).

4.1. Methods for SSAM Amplification of mir-9

Step 1—Synthesis of the complementary strand of the FON. The sequence of the FON; Bio/AAC GAG GAA TGC GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGA TCA TAC AGC TAG ATA A. 1 femtomole (fm) of FON and 100 nanograms (ng) of total RNA were mixed with following reagents: 4 µl of 5x reverse transcription buffer; 2 µl dNTPs (10 mM); 1 µl dithiothreitol (DTT; 10 mM). The reaction mixture was incubated in a thermal cycler at 72 °C for 2 minutes (min) and then the temperature was decreased to 55 °C. 1 µl of RNase inhibitor (SUPERaseIn, Life Technologies, Carlsbad, CA; 20 U) and 1 µl of Superscript III (Life Technologies; 200 U) were added into reaction mixture. The total volume was adjusted to 20 µl with 18.2 mega Ohm RNase-free water (Nanopure Diamond, Dubuque, IA) and the reaction mixture was further incubated at 55 °C for 30 min.

Step 2—Digestion of the excess FON. After first strand synthesis, Exonuclease I (20 U; New England BioLabs, Ipswich, MA) was directly added into the sample mixture and incubated at 37 °C for a hour (h), followed by heating at 80 °C for 20 min to inactivate the enzyme.

Step 3—Attaching the SON to the FON; The sequence of SON of mir-9; GGC GCG CCG AAT TCT TCA TAC TCA TAC GTT ATC TAG CTT CTT TGG TTA TCT AGC TTC TTT GGT TAT CTA GCT. The SON was incubated at 84 °C for 2 min and cooled on wet ice. 1 fm SON and 1 µl RNase H (10 U) was added into the reaction mix and incubated at 37 °C for 30 min. 1 µl 3'–5' exonuclease negative Klenow fragment (5 U) was added and the reaction mix was incubated at 37 °C for 30 min. The reaction was stopped by incubating the reaction mix at 75 °C for 20 min to inactivate the enzymes.

Step 4—Amplifying the signature sequence of the target miRNA using IVT. The reaction mix was purified and concentrated using Vivaspin 500 column (Sartorius, Edgewood, NY). To purify the sample, the entire sample was transferred into a Vivaspin 500 column (30,000 MWCO PES), add 500 µl was added water the column. The column was spun at 15,000 g for 6 min. Samples were collected into microfuge tubes and the volume adjusted to 15 µl. For IVT, to each 7.5 µl sample, 4 µl of 5x IVT buffer, 1 µl (2.5 mM) 3 nucleotide triphosphates (NTPs; A, C, G), 1 µl UTP (100 µM), 0.5 µl RNasin (10 U), 4 µl (80 µCi) ³³P (GE Healthcare, Piscataway, NJ), and 1 µl T7 RNA polymerase (Epicentre, Madison, WI; 1000 U) was added, and the reaction mix was incubated at 37 °C for 4 h. Radiolabeled probes were hybridized to custom-designed oligonucleotide arrays without further purification as described previously [47, 48].

4.2. Generation of a mir-9 Array

The SON of mir-9 was amplified *via* PCR. Forward primer: AGC TAG ATA ACC AAA GAA. Reverse primer: GGC CAG TGA ATT GTA ATA CGA. The PCR reaction mixture was comprised of 1 fm of SON, 0.1 nm of each forward and reverse primer, 50 µl of 2x AmpliTaq Gold PCR Master Mix (Life Technologies), in a final volume of 100 µl. The amplification conditions were: 95 °C 3 min, followed by 50 °C 45 seconds (sec), 68 °C at 1 min for 40 cycles. The concentration of the PCR product was visualized *via* agarose gel electrophoresis, and 0.1 µg of PCR product was spotted on nylon membranes. Membranes

were cut to the appropriate size and soaked in 2X saline-sodium citrate (SSC) buffer prior to arraying. PCR products were diluted in 2X SSC buffer and denatured at 95 °C for 5 min. The denatured PCR product was spotted on the membrane using a slot blot apparatus (Schleicher & Schuell, Keene, NH). Spotted arrays were baked at 80 °C in a vacuum oven for 2 h to adhere the nucleic acids to the membrane for downstream applications.

4.3. Detection of the mir-9 Amplification Products

Detection of the amplification products was performed by hybridization of the amplified sample to oligonucleotides immobilized on a solid support, nylon membranes (Fig. 4). Arrays were prehybridized (2 h) and hybridized (14–16 h) in a solution consisting of 6X saline sodium phosphate EDTA buffer (SSPE), 5x Denhardt's solution, 50% formamide, 0.1% sodium dodecyl sulfate (SDS), and denatured salmon sperm DNA (200 µg/ml) at 42 °C in a rotisserie oven. Following hybridization, arrays were washed sequentially in 2X SSC/0.1% SDS, 1X SSC/0.1% SDS and 0.5X SSC/0.1% SDS for 15 min each at 37 °C. Arrays were placed in a phosphor screen for 24 h and developed on a phosphor imager (GE Healthcare). Hybridization signal intensity was determined by utilizing ImageQuant TL array analysis program (GE Healthcare) (Fig. 5).

5. CURRENT & FUTURE DEVELOPMENTS

The field of miRNA study is in its infancy. The role(s) miRNA and other regulatory ncRNA defects play in a wide range of disciplines, including oncology and neurodegenerative biology, is just beginning to be appreciated. The stated goal of the SSAM technology is to develop and refine a methodology for miRNA and/or small polynucleotide sequence detection and quantitation that will be useful for biomarker studies and functional genetic analyses. The SSAM methodology enables downstream genetic manipulations including miRNA microarray and real-time qPCR based applications for biomarker discovery science. Furthermore, the SSAM technology has tremendous potential for discrimination and quantitation of miRNAs and small polynucleotide sequences from minute tissue samples, which will have widespread technical and theoretical applications in all fields of biology. Importantly, we demonstrate the ability to analyze multiple miRNAs in one SSAM amplification reaction, a decided improvement over currently available conventional technologies. Future developments include the use of the SSAM technology for discovery-based science for unknown species, including but not limited to miRNAs, siRNAs, shRNAs, lncRNAs, and other regulatory ncRNAs that may have commercial and/or research and development possibilities in human samples as well as in relevant animal and cellular models.

Acknowledgments

We thank Ashley Hart, Darrell Belch, and David Nocilly for assistance with protecting the intellectual property in 'Methods and compositions for amplification and detection of microRNAs', US Patent 8,580,494. We also thank Judah A. Beilin, B.A. for assistance with graphics.

References

1. Green, MR.; Sambrook, J. *Molecular Cloning: A Laboratory Manual*. 4. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 2012.
2. Gygi SP, Rochon Y, Franza BR, Aebersold R. Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol*. 1999; 19:1720–30. [PubMed: 10022859]
3. Venter JC, Adams MD, Myers EW, et al. The sequence of the human genome. *Science*. 2001; 291:1304–51. [PubMed: 11181995]
4. Venkatesh B, Kirkness EF, Loh YH, et al. Ancient noncoding elements conserved in the human genome. *Science*. 2006; 314:1892. [PubMed: 17185593]
5. Kugel JF, Goodrich JA. Non-coding RNAs: key regulators of mammalian transcription. *Trends Biochem Sci*. 2012; 37:144–51. [PubMed: 22300815]
6. Harold D, Abraham R, Hollingworth P, et al. Genome-wide association study identifies variants at *CLU* and *PICALM* associated with Alzheimer's disease. *Nat Genet*. 2009; 41:1088–93. [PubMed: 19734902]
7. Arora S, Rana R, Chhabra A, Jaiswal A, Rani V. miRNA-transcription factor interactions: a combinatorial regulation of gene expression. *Mol Genet Genomics*. 2013; 288:77–87. [PubMed: 23334784]
8. Bhaskaran M, Mohan M. MicroRNAs: history, biogenesis, and their evolving role in animal development and disease. *Vet Pathol*. 2013; 51:759–74. [PubMed: 24045890]
9. Tijsterman M, Plasterk RH. Dicers at RISC; the mechanism of RNAi. *Cell*. 2004; 117:1–3. [PubMed: 15066275]
10. Soifer HS, Rossi JJ, Saetrom P. MicroRNAs in disease and potential therapeutic applications. *Mol Ther*. 2007; 15:2070–9. [PubMed: 17878899]
11. Kosik KS, Krichevsky AM. The elegance of the microRNAs: a neuronal perspective. *Neuron*. 2005; 47:779–82. [PubMed: 16157272]
12. Carrington JC, Ambros V. Role of microRNAs in plant and animal development. *Science*. 2003; 301:336–8. [PubMed: 12869753]
13. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*. 1993; 75:843–54. [PubMed: 8252621]
14. Kozomara A, Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res*. 2011; 39:D152–D7. [PubMed: 21037258]
15. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res*. 2014; 42:D68–D73. [PubMed: 24275495]
16. Krek A, Grun D, Poy MN, et al. Combinatorial microRNA target predictions. *Nat Genet*. 2005; 37:495–500. [PubMed: 15806104]
17. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*. 2009; 19:92–105. [PubMed: 18955434]
18. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*. 2005; 120:15–20. [PubMed: 15652477]
19. Ritchie W, Rasko JE. Refining microRNA target predictions: sorting the wheat from the chaff. *Biochem Biophys Res Commun*. 2014; 445:780–4. [PubMed: 24525131]
20. Beveridge NJ, Cairns MJ. MicroRNA dysregulation in schizophrenia. *Neurobiol Dis*. 2012; 46:263–71. [PubMed: 22207190]
21. Lukiw WJ, Andreeva TV, Grigorenko AP, Rogaev EI. Studying micro RNA function and dysfunction in Alzheimer's disease. *Front Genet*. 2012; 3:327. [PubMed: 23390425]
22. Meister B, Herzer S, Silahatoglu A. MicroRNAs in the hypothalamus. *Neuroendocrinology*. 2013; 98:243–53. [PubMed: 24080764]
23. Dogini DB, Avansini SH, Vieira AS, Lopes-Cendes I. MicroRNA regulation and dysregulation in epilepsy. *Front Cell Neurosci*. 2013; 7:172. [PubMed: 24109432]
24. Kuss AW, Chen W. MicroRNAs in brain function and disease. *Curr Neurol Neurosci Rep*. 2008; 8:190–7. [PubMed: 18541114]

25. Maffioletti E, Tardito D, Gennarelli M, Bocchio-Chiavetto L. Micro spies from the brain to the periphery: new clues from studies on microRNAs in neuropsychiatric disorders. *Front Cell Neurosci.* 2014; 8:10.3389. [PubMed: 24478632]
26. Garza-Manero S, Pichardo-Casas I, Arias C, Vaca L, Zepeda A. Selective distribution and dynamic modulation of miRNAs in the synapse and its possible role in Alzheimer's disease. *Brain Res.* 2014; 8993:1536–9.
27. Kosik KS. The neuronal microRNA system. *Nat Rev Neurosci.* 2006; 7:911–20. [PubMed: 17115073]
28. Kindler S, Kreienkamp HJ. Dendritic mRNA targeting and translation. *Adv Exp Med Biol.* 2012; 970:285–305. [PubMed: 22351061]
29. Schratt G. microRNAs at the synapse. *Nat Rev Neurosci.* 2009; 10:842–9. [PubMed: 19888283]
30. Schratt GM, Tuebing F, Nigh EA, et al. A brain-specific microRNA regulates dendritic spine development. *Nature.* 2006; 439:283–9. [PubMed: 16421561]
31. Kye MJ, Liu T, Levy SF, et al. Somatodendritic microRNAs identified by laser capture and multiplex RT-PCR. *RNA.* 2007; 13:1224–34. [PubMed: 17592044]
32. Sun AX, Crabtree GR, Yoo AS. MicroRNAs: regulators of neuronal fate. *Curr Opin Cell Biol.* 2013; 25:215–21. [PubMed: 23374323]
33. Roeske-Koerner B, Stappert L, Koch P, Brustle O, Borghese L. Pluripotent stem cell-derived somatic stem cells as tool to study the role of microRNAs in early human neural development. *Curr Mol Med.* 2013; 13:707–22. [PubMed: 23642053]
34. Kane NM, Thrasher AJ, Angelini GD, Emanuelli C. Concise review: MicroRNAs as modulators of stem cells and angiogenesis. *Stem Cells.* 2014; 32:1059–66. [PubMed: 24449004]
35. Elramah S, Landry M, Favereaux A. MicroRNAs regulate neuronal plasticity and are involved in pain mechanisms. *Front Cell Neurosci.* 2014; 8:10.3389. [PubMed: 24478632]
36. Fabbri M, Calore F, Paone A, Galli R, Calin GA. Epigenetic regulation of miRNAs in cancer. *Adv Exp Med Biol.* 2013; 754:137–48. [PubMed: 22956499]
37. Farazi TA, Hoell JI, Morozov P, Tuschl T. MicroRNAs in human cancer. *Adv Exp Med Biol.* 2013; 774:1–20. [PubMed: 23377965]
38. Kita Y, Vincent K, Natsugoe S, Berindan-Neagoe I, Calin GA. Epigenetically regulated microRNAs and their prospect in cancer diagnosis. *Expert Rev Mol Diagn.* 2014; 10:1–11.
39. Ginsberg SD, Mimics K. Functional genomic methodologies. *Progress Brain Res.* 2006; 158:15–40.
40. Ginsberg SD, Alldred MJ, Che S. Gene expression levels assessed by CA1 pyramidal neuron and regional hippocampal dissections in Alzheimer's disease. *Neurobiol Dis.* 2012; 45:99–107. [PubMed: 21821124]
41. Ginsberg SD. RNA amplification strategies for small sample populations. *Methods.* 2005; 37:229–37. [PubMed: 16308152]
42. Biggar KK, Wu CW, Storey KB. High-throughput amplification of mature microRNA in uncharacterized animal models using polyadenylated RNA and stem-loop RT-PCR. *Anal Biochem.* 2014; 2697:251–6.
43. Benes V, Castoldi M. Expression profiling of microRNA using real-time quantitative PCR, how to use it and what is available. *Methods.* 2010; 50:244–9. [PubMed: 20109550]
44. Gorreta F, Carbone W, Barzaghi D. Genomic profiling: cDNA arrays and oligoarrays. *Methods Mol Biol.* 2012; 823:89–105. [PubMed: 22081341]
45. Witwer KW. Data submission and quality in microarray-based microRNA profiling. *Clin Chem.* 2013; 59:392–400. [PubMed: 23358751]
46. Saba R, Booth SA. Target labelling for the detection and profiling of microRNAs expressed in CNS tissue using microarrays. *BMC Biotechnol.* 2006; 6:47. [PubMed: 17164008]
47. Che, S.; Ginsberg, SD. RNA amplification methodologies. In: McNamara, PA., editor. *Trends in RNA Research*. Hauppauge: Nova Science Publishing; 2006. p. 277–301.
48. Che S, Ginsberg SD. Amplification of transcripts using terminal continuation. *Lab Invest.* 2004; 84:131–7. [PubMed: 14647400]

49. Ho YK, Xu WT, Too HP. Direct quantification of mRNA and miRNA from cell lysates using reverse transcription real time PCR: a multidimensional analysis of the performance of reagents and workflows. PLoS ONE. 2013; 8:e72463. [PubMed: 24039771]
50. Pena-Llopis S, Brugarolas J. Simultaneous isolation of high-quality DNA, RNA, miRNA and proteins from tissues for genomic applications. Nat Protoc. 2013; 8:2240–55. [PubMed: 24136348]
51. Lundin KE, Hojland T, Hansen BR, et al. Biological activity and biotechnological aspects of locked nucleic acids. Adv Genet. 2013; 82:47–107. [PubMed: 23721720]
52. Soe MJ, Dufva M, Holmstrom K. Detection of small noncoding RNAs by in situ hybridization using probes of 2'-O-Methyl RNA + LNA. Methods Mol Biol. 2014; 1173:113–21. [PubMed: 24920364]
53. Alldred MJ, Che S, Ginsberg SD. Terminal continuation (TC) RNA amplification without second strand synthesis. J Neurosci Methods. 2009; 177:381–5. [PubMed: 19026688]
54. Ginsberg SD. Transcriptional profiling of small samples in the central nervous system. Methods Mol Biol. 2008; 439:147–58. [PubMed: 18370101]
55. Ginsberg SD, Lee SH, Irwin DJ, et al. qPCR analysis of 3-repeat tau (3Rtau) and 4-repeat tau (4Rtau) isoforms in Alzheimer's disease. Proc Soc Neurosci. 2014 in press.
56. Che S, Andreadis A, Petkova E, Ginsberg SD. Analysis of individual 3-repeat tau (3Rtau) and 4-repeat tau (4Rtau) isoforms in postmortem human entorhinal cortex *via* a qPCR-based assay. Proc Soc Neurosci. 2012; 38:50.16.

Fig. 1. Schematic illustrating the signature sequence generation process for amplification of miRNAs/small polynucleotides using SSAM technology.

Key: miRNA' deoxyribo miRNA complimentary sequence. miRNA deoxyribo miRNA sequence. ribo miRNA sequence. DNA fragment in SON with identical sequence to the target miRNA.

Fig. 2.

Depiction of the clearance of free single stranded FON with enzymatic digestion by exonuclease I before the addition of SONs.

Fig. 3. Illustration depicting the methods to clear excess residual single stranded FONs from samples. Biotin labeled single stranded FONs become double stranded in the present of target miRNAs and a DNA polymerase which can be digested by a restriction endonuclease.

Fig. 4. Schematic demonstrating double stranded FON clearance. A biotin labeled strand of a partially double stranded FON is replaced in the presence of target miRNA and a DNA polynuclease. Residual FONs are cleared by SA_v conjugated beads whereas FONs present in DNA/RNA hybrid molecule remain in the sample.

Fig. 5. Identifying mir-9 using the SSAM procedure. mir-9 miRNA can be detected by the SSAM technology with minimal background (**A**) Proof of concept for linearity of the SSAM procedure. (**B**) Femtomolar concentrations of mir-9 miRNA were assayed on a custom-designed miRNA array platform. The hybridization signal intensity is demonstrated following a 3-fold dilution of initial input of mir-9 (10, 3.3, and 1.1 fm) in a signature sequence reaction with 50 fm each of the FON and SON.