Sensitive and specific miRNA detection method using SplintR Ligase

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ABSTRACT

We describe a simple, specific and sensitive microRNA (miRNA) detection method that utilizes Chlorella virus DNA ligase (SplintR® Ligase). This two-step method involves ligation of adjacent DNA oligonucleotides hybridized to a miRNA followed by real-time quantitative PCR (qPCR). SplintR Ligase is 100X faster than either T4 DNA Ligase or T4 RNA Ligase 2 for RNA splinted DNA ligation. Only a 4–6 bp overlap between a DNA probe and miRNA was required for efficient ligation by SplintR Ligase. This property allows more flexibility in designing miRNA-specific ligation probes than methods that use reverse transcriptase for cDNA synthesis of miRNA. The qPCR SplintR ligation assay is sensitive; it can detect a few thousand molecules of miR-122. For miR-122 detection the SplintR qPCR assay, using a FAM labeled double quenched DNA probe, was at least 40× more sensitive than the TaqMan assay. The SplintR method, when coupled with NextGen sequencing, allowed multiplex detection of miRNAs from brain, kidney, testis and liver. The SplintR qPCR assay is specific; individual let-7 miRNAs that differ by one nucleotide are detected. The rapid kinetics and ability to ligate DNA probes hybridized to RNA with short complementary sequences makes SplintR Ligase a useful enzyme for miRNA detection.

INTRODUCTION

MicroRNAs (miRNAs) were first described as regulators of developmental timing in *Caenorhabditis elegans* by Ambros and Ruvkun [1,2]. The finding that lin-4 coded for a 21 nucleotide (nt) long RNA complementary to the 3′ UTR of lin-14 mRNA suggested the miRNAs alter mRNA translation by RNA base pairing. The proposed biological roles of miRNAs expanded when the let-7 miRNA, originally described in *C. elegans*, was detected in a diverse range of animals, from nematodes to mammals [3]. Most eukaryotic organisms express hundreds of miRNAs, each modulating the translation of multiple messenger RNAs. Regulation of gene expression by miRNA is critical for cell development and differentiation. Specific patterns of miRNA expression serve as biomarkers for a diverse range of diseases [4]. Accurate detection and quantitation of miRNAs are critical both for understanding their biological roles and for developing rigorous diagnostic tests for a variety of cancers [5]. Current methods for characterization of miRNAs fall into four general classes: miRNA-specific hybridization, next-generation sequencing, qPCR amplification/detection, and ligation and detection of complementary DNA probes.

The initial miRNA detection studies used Northern blots that identified membrane bound miRNAs by hybridization to a labeled miRNA-specific DNA probe [6,7]. The early methods were not sensitive because of difficulty in binding short RNAs to membranes. This problem was addressed by modifications to the initial Northern blot strategy. One method lengthens the RNA by ligation of ^{32}P-labeled DNA to the end of the miRNA using a DNA splint. This internal radioactive label is resistant to phosphatase, and the ligated product can be detected after electrophoresis [8]. Another hybridization method uses the p19 RNA binding protein to selectively bind a miRNA:RNA-probe duplex, but p19 does not bind unhybridized ssRNA probe [9]. The enrichment of miRNA:RNA-probe duplex by p19 can be detected by gel electrophoresis or with a nanopore that detects individual miRNAs [10]. Microarrays and the Nanostring nCounter are two methods that use enzymatic labeling of the miRNA prior to hybridization and detection. The nCounter method requires ligation of miRTag oligonucleotide to the 3′ of the miRNA using a second oligonucleotide as a bridge to generate an RNA:DNA duplex for ligation. Multiplex detection of specific miRNAs is achieved by decoding the linear arrangement of different colored fluorescent dyes [11]. Microarray methods typically label RNA via ligation of biotin or dye labeled nucleotide followed by hybridization to an array. The labeling is not miRNA specific and there is variation in the efficiency of labeling the 3′ end of the RNA [12].

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In recent years, the discovery of novel miRNAs has been greatly accelerated by the adoption of high throughput next-generation sequencing of small RNAs; as of 2014 there were over 28,000 miRNAs from 223 species in miRBase (13). Though sequencing is very efficient for miRNA discovery, these methods do not allow accurate quantification of miRNA expression levels due to sequencing bias. The bias derives primarily in ligation of single-stranded adapters to miRNA, with some sequences ligating with greater efficiency than others (6,14). Experiments with a defined library of synthetic miRNAs in known ratios have identified greater than a 1000X preferential sequencing of some miRNA sequences over others. It has been proposed that ligation bias is derived from the secondary structure formed by the miRNAs and their adapters, with some structures ligating much more efficiently than others. (15–17).

The preferred method for quantitative detection of specific nucleic acid sequences is typically a quantitative PCR (qPCR) based assay. However, the short size of miRNAs complicates their detection: since miRNAs are only 22 nucleotides long they are too short to use two convergent DNA primers for efficient amplification by reverse transcriptase (RT-PCR). One solution uses poly(A) polymerase to lengthen the miRNA through the addition of a poly(A) tail to the 3′ end of the miRNA, allowing the use of oligo dT as a primer for cDNA synthesis by reverse transcriptase (18). There are limitations in using poly(A) tailing for miRNA detection. *In vitro* studies indicate significant reduction in poly(A) tailing of miRNAs if the RNA has a terminal stem loop structure (19). Polyadenylation of miRNAs is also inhibited by the presence of 2'-O methylation on the 3′ terminal ribose (20). The 2'-O-Me is a common feature of plant miRNAs and Piwi RNAs (21). Limitations also exist for a second approach that uses a DNA hairpin with a short 3′ extension complementary to the 3′ end of the target miRNA as a primer for cDNA synthesis by reverse transcriptase. After cDNA synthesis the miRNA can be detected by qPCR using miRNA-specific DNA primers for amplification. Since the miRNA is only 22 nt in length there are constrains on the length and location of these specific primers. Efficiency of amplification depends upon both GC content and extent of the overlap with the miRNA. Secondary structure of the miRNA can also introduce bias in cDNA synthesis in both methods.

The method used to monitor qPCR amplification can further influence specificity and sensitivity of miRNA detection. Two commonly used approaches are SYBR Green® and miRNA-specific TaqMan® probes. SYBR Green binds to any amplified dsDNA, so it does not discriminate between any amplified DNA. TaqMan probes have greater specificity because they hybridize to a specific sequence. TaqMan probe contains a fluorophore and a single quencher. The hybridized probe is degraded by the 5′ to 3′ exonuclease activity of the DNA polymerase used during PCR amplification, which separates the fluorophore from the quencher. The quenched probes improve the specificity of the assay allowing real time measurement of the specific amplified DNA (22).

Ligation of DNA probes followed by DNA amplification offers a fourth approach for miRNA detection. Unlike the DNA hairpin primer, sequences for ligation of DNA probes

are not restricted to the 3′ end of the miRNA. This allows longer probes to be designed that form stable hybrids which are less susceptible to disruption by reaction temperature or RNA structure. One embodiment of this approach is the use of a 'padlock' probe followed by rolling circle amplification. The ends of a single stranded DNA probe are hybridized to a specific miRNA and then ligated by T4 DNA ligase to generate a circular ssDNA. This ligated circle can be detected with high sensitivity through rolling circle amplification (23). Another method uses T4 DNA ligase to join miRNA-specific DNA probes of different length to detect multiple miRNAs. The ligated probes are amplified by PCR and detected by gel electrophoresis. The size of the amplified products correlates with a specific miRNA (24). A ligation based method for messenger RNA detection is called RASL-seq, for **R**NA-mediated oligonucleotide **A**nnealing, **S**election, and **L**igation with Next-Gen sequencing. This combines ligation with PCR amplification followed by next-generation sequencing. The number of the DNA sequence reads reflects the abundance of the different mRNAs (25).

These previously described miRNA ligation-based detection methods use T4 DNA Ligase, despite its highly inefficient ligation of DNA in an RNA:DNA hybrid. A superior enzyme for this type of ligation is Chlorella virus DNA ligase (sold commercially as SplintR Ligase). Although the Chlorella virus DNA ligase has been extensively characterized for ligation of a nick in dsDNA (26,27), only recently has it been found to efficiently ligate two DNA oligos splinted by RNA (28). Compared to T4 DNA Ligase, Chlorella virus DNA ligase has a faster maximum turnover rate ($>20\times$) and a much lower K_m (1 nM versus 300 nM for T4 DNA Ligase) for the RNA:DNA substrates. The relatively poor binding of the T4 enzyme to the RNA:DNA hybrid often results in abortive ligation with the accumulation of the adenylated DNA oligonucleotides in preference to productive ligation (28). This observation motivated us try to develop a miRNA detection assay using SplintR ligase. Here, we report a two-step miRNA detection method: ligation of miRNA-specific DNA oligonucleotides followed by SplintR followed by amplification and detection in a qPCR-based assay. The SplintR method can detect single base differences in related members of the let-7 miRNA family. We also compare this new method to TaqMan and further improve it by using a double-quenched fluorescent DNA probe containing a ZEN quencher (29). The assay is sensitive, capable of detecting <1 amol of miR-122 by qPCR using a double-quenched flourescent DNA probe. The SplintR assay was 40-fold more sensitive than a TaqMan probe designed to detect the same miRNA.

MATERIALS AND METHODS

Ligases and Polymerases

All ligases and buffers were obtained from New England Biolabs, Inc (NEB), Ipswich, MA, USA. The concentrations of the selling stocks of the enzymes were: T4 DNA Ligase high concentration (45 pmol/μl), T4 RNA Ligase 2 (6.6 pmol/μl) and SplintR Ligase (10.5 pmol/μl). To compare ligation activites, 10 pmol of enzyme was used in each ligation reaction according to the manufactures protocol. T4

DNA Ligase and SplintR Ligase reactions were performed in 1× T4 DNA Ligase buffer. This buffer has the same composition as SplintR reaction buffer (NEB). T4 RNA Ligase 2 reactions were performed in 1X RNA Ligase 2 buffer (NEB). The microRNA reverse transcription kit and Taq-Man universal PCR master mix were purchased from Life Technologies (Carlsbad, CA, USA). The One Taq Hot Start 2X PCR Master Mix was obtained from NEB.

DNA and RNA

Escherichia coli rRNA was obtained from New England Biolabs. Rat liver total RNA, mouse kidney total RNA and rat testis total RNA were purchased from Molecular Research Center, Cincinnati, Ohio. DNA or RNA oligonucleotides were all purchased from Integrated DNA Technologies (IDT) unless otherwise indicated. The oligonucleotides were dissolved in water to make concentrated stock solutions of 50 or 100 mM.

miR-122 scanning DNA probes

Ten pairs of DNA oligonucleotides, complementary to miR-122, were used to determine the minimum length of an RNA:DNA hybrid required for ligation. The probe A set has a 5′ FAM label and the probe B set has a 5′ phosphate that allows ligation to probe A. The number of the probe represents the nucleotides complementary to miR-122 as shown in Figure 1. The sequence of each pair of probes is listed in the Supplementary Data.

miR-122 qPCR primers

qA10: 5′ CTCGACCTCTCTATGGGCAGTCACGACC AAACACCAT
   qB12: 5′ pTGTCACACTCCACTGAGTCGGAGAC ACGCAGGGCTTAA
   Forward primer, miR-122 fp: 5′ GCTCGACCTCTCTA TGGGC
   Reverse primer, miR-122 rp: 5' TTAAGCCCTGCGTG TCTCC

miR-122 single-quenched probe

miR-122 sqp: 5′ FAM CACGACCAAACACC MGB/NF Q. The 3′ quencher is a minor groove binder (MGB) non-fluorescent quencher (NFQ). The TaqMan miR-122 probe was purchased from Life Technologies.

miR-122 double-quenched probe

miR-122 dqp: 5′ FAM/TCACGACCA/ZEN/AACACCAT TGTCACACTCCA/3′ IBFQ.
   FAM is fluorescein, ZEN^{TM} is an internal quencher located 9 bases from FAM, IBFQ is Iowa Black fluorescence quencher that has an absorbance peak at 531 nm. The probe was synthesized by IDT.

miR let-7 family synthetic RNA

Nucleotide differences between let-7a and the other members of the let-7 family are indicated by a bold font.

Let-7a: 5′ pU G A G G U A G U A G G U U G U A U A G U U
   Let-7b: 5′ pU G A G G U A G U A G G U U G U **G** U **G** G U U
   Let-7c: 5′ pU G A G G U A G U A G G U U G U A U **G** G U U
   Let-7d: 5′ pA G A G G U A G U A G G U U G **C** A U A G U U
   Let-7e: 5′ pU G A G G U A G **G** **G** A G G U U G U A U A G U U
   Let-7f: 5′ pU G A G G U A G U A G **A** U U G U A U A G U U
   Let-7g: 5′ pU G A G G U A G U A G **U** U U G U A **C** A G U U
   Let-7i: 5′ pU G A G G U A G U A G **U** U U G U **G** **C** **U** G U U

Let-7b oligos for gel assay

Let-7b DNA probe gA5: 5′ 6FAM CCTCTCTATGGGCA GTCGGTGAAACCA
   Let-7b DNA probe gB17: 5′ pCACAACCTACTACCT CACTGAGTCGGAGACACGCAGGG

Let-7c oligos for gel assay

Let-7c DNA probe gA4: 5′ FAM CCTCTCTATGGGCA GTCGGTGAAAAACC
   Let-7c DNA probe gB18: 5′ pATACAACCTACTACC TCACTGAGTCGGAGACACGCAGGG

Let-7g oligos for gel assay

Let-7g DNA probe gA6: 5′ FAM CCTCTCTATGGGCA GTCGGTGAAACTGT
   Let-7g DNA probe gB16: 5′ pACAAACTACTACCTC ACTGAGTCGGAGACACGCAGGG

Let-7b oligos for qPCR assay

DNA probe let-7b qA5: 5′TGTCTGGTCAGAATCACC TCCTCGTCATGGGCAGGTACGGTGCAACCA3′
   DNA probe let-7b qB17:
   5′p CACAACCTACTACCTCACTGAGTTGGAGAC ACGCAGAA 3′

Let-7c oligos for qPCR assay

DNA probe let-7c qA4: 5′TGTCTGGTCAGAATCACC TCCTCGTCATGGGCAGGTACGGTGCAACC 3′
   DNA probe let-7c qB18:
   5′pATACAACCTACTACCTCACTGAGTTGGAG ACACGCAGAA 3′

Let-7g oligos for qPCR assay

DNA probe let-7g qA6:
   5′TGTCTGGTCAGAATCACCTCCTCGTCATGG GCAGGTACGGTGCAACTGT 3′
   DNA probe let-7g qB16:
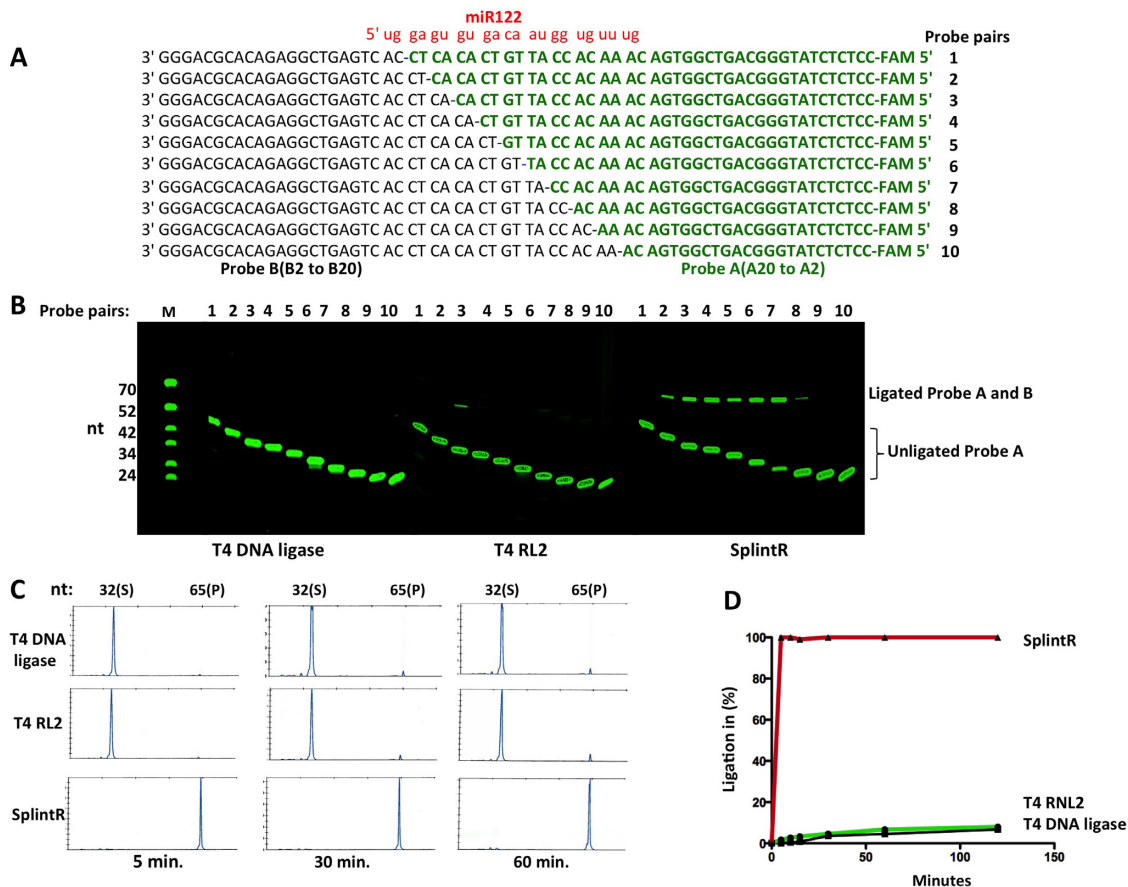   5′pACAAACTACTACCTCACTGAGTTGGAGAC ACGCAGAA 3′

Figure 1. Comparison of T4 DNA Ligase, T4 RNA Ligase 2 and SplintR Ligase for their ability to join different length DNA probes hybridized to miR-122. (**A**) Pairs of DNA oligonucleotides complementary to miR-122 were used to determine the minimum DNA:RNA overlap required for ligation. The miR-122 sequence (red) is complementary to probe A (green) and probe B (black). Probe A and probe B are designed to scan the miRNA sequence in two base increments. Probe A has a 5′ FAM label and probe B has a 5′ phosphate that allows ligation. Each pair of DNA probes is named by the number of nucleotides (nt) that are complementary to the 22 nt in miR-122, for example probe A8 has 8 nt and B14 has 14 nt complementary to miR-122. (**B**) The ligation of FAM labeled probe A to probe B is visualized by a fluorescent scan after electrophoresis on a 15% TBE urea acrylamide gel. Probe B is not observed because only probe A has a FAM label. Since there is more probe A than miR-122 each reaction has both ligated and unligated probe. Lane M, at the left of the gel, denotes the size in nucleotides of the FAM labeled ssDNA marker. The specific components and reaction conditions are described in Materials and Methods. (**C**) The ligation of DNA probes hybridized to miR-122 by SplintR Ligase, T4 DNA Ligase or T4 RNA Ligase 2 (T4 RL2) (indicated on the left) at different incubation times was measured by capillary electrophoresis (CE) fragment analysis. The traces are shown for 5, 30 and 60 minute ligations of probes gA10 and gB12 hybridized to miR-122. Position of the 32 nt substrate (32(S)) and the 65 nt ligated product (65(P)) are indicated on the top of the panels. Reaction conditions are listed in Materials and Methods. (**D**) Ligation time course for the three enzymes, SplintR (red), T4 DNA ligase (black) and T4 RNA ligase 2 (green) was measured by CE. The percent of ligation is indicated on the left of the graph.

Let-7 stacking oligo complementary to probe A

5′ TCACCGACTGCCCATAGAGAGG

The stacking oligo is complementary to the conserved probe A sequence adjacent to the 3′ end of the let-7 miRNAs.

Double-quenched let-7 fluorescent DNA probe

let-7 dqp: 5′ FAM-CGTCATGGG-ZEN-CAGGTACGGTGCAAC-31ABkFQ 3′

The sequence of the let-7 double quenched qPCR probe is contained in the conserved region of probe A and has the three nucleotides at the 3′ end complementary to let-7 miRNAs. The last three nucleotides of the double-quenced let-7 fluorescent probe are AAC, which is complementary to the 3′ end of all members of the let-7 family, which end in GUU.

Let-7 qPCR primers

qPCR forward primer, let-7 fp: 5′ GTCTGGTCAGAATCACCTC

qPCR reverse primer, let-7 rp: 5′ TTCTGCGTGTCTCCAACTCA

The qPCR forward and reverse primers were used to amplify the ligated let-7b, let-7c and let-7g probes.

PAGE-based ligation assay

To determine the minimum length of RNA:DNA duplex required for ligation we used pairs of DNA oligonucleotides complementary to miR-122 to scan the miRNA. The following conditions were used for ligation. DNA probes A and B were annealed to miR122; 1 μl (50 pmol) of DNA probe A, 1 μl (50 pmol) of DNA probe B and 1 μl (28 pmol) of miR122 were mixed in 1× T4 DNA Ligase buffer in a to-

tal volume of 10 µl. The oligos were heated to 95°C for 5 min in a heat block then cooled down slowly to 22°C. The ligation reactions contained, 5 µl of the annealed probes, 1 or 2 µl (10 pmol) of either T4 DNA Ligase, T4 RNA Ligase 2 or SplintR Ligase, 0.5 µl of 10× ligase buffer and H_2O in a final volume of 10 µl. The T4 DNA Ligase and SplintR Ligase reactions were incubated at 16°C for 1 h. The T4 RNA Ligase 2 reactions were incubated at 37°C for one hour. A 2 µl aliquot from the ligation reaction was mixed with 3X formamide denaturing buffer (NEB), diluted with H_2O to 20 µl, heated at 95°C for 5 min and then loaded onto a 15% TBE urea gel (Invitrogen). A FAM-labeled single stranded DNA ladder was also loaded as a size marker. Gels were scanned with Typhoon 9400 scanner and visualized with ImageQuant TL software (GE healthcare).

To detect individual let-7 miRNAs, isoform specific DNA probes were used. The three pairs of probes are: let-7b (gA5:gB17), let-7c (gA4:gB18) and let-7g (gA6:gB16). Probes A for let-7b and let-7c have a short complementary sequence of 4 or 5 nt with the miRNA. To enhance ligation a stacking oligo, complementary to conserved region of probe A, was included in the hybridization. Each pair of DNA probes were hybridized separately to each one of the eight let-7 isoforms: probe A (5 pmol), probe B (20 pmol), let-7 miRNA isoform (16 pmol) and stacking DNA oligo (10 pmol) in 1× T4 DNA Ligase buffer in a final volume of 10 µl. The denaturation, hybridization and gel analysis are described in the preceding paragraph.

Ligation analysis by capillary electrophoresis (CE)

To obtain a quantitative measurement of ligation, capillary electrophoresis (CE) analysis was used (28). An annealing reaction master mix was prepared by mixing 60 µl (9.36 nmol) of miR-122, 30 µl (3 nmol) of DNA probe A8, 120 µl (12 nmol) of DNA probe B14 and 30 µl of 10× T4 DNA ligase buffer in 240 µl total volume then denatured and hybridized as described above. The ratio of miR122, DNA probe A8 and DNA probe B14 in ligation reaction was 39 pmol/µl : 12.5 pmol/µl : 50 pmol/µl. Three separate ligation master mixes were made for the three ligases. Each ligation reaction contained 2 µl of annealing mix, 1 µl of 10× T4 DNA Ligase buffer (for T4 DNA Ligase and SplinR Ligase) or 1 µl of 10× T4 RNA Ligase buffer (for T4 RNA Ligase 2), 10 pmol of ligase and H_2O to a total volume of 10 µl. Six separate reactions were made for each enzyme and incubated for 5, 10, 15, 30, 60 and 120 min. Ligation reactions containing SplintR Ligase and T4 DNA Ligase were incubated at 16°C, reactions containing T4 RNA Ligase 2 at 37°C. Ligation reactions were stopped by adding in an equal volume of 2× stop buffer (50 mM EDTA, 0.1% Triton) and frozen at −20°C. A one tenth aliquot (2 µl) was removed from each ligation, diluted 100× with water and analyzed by CE on an Applied Biosystems 3730×1 Genetic Analyzer (96 capillary array) as previously described (28).

SplintR qPCR miRNA detection

miR-122 assay. Synthetic miR-122 oligonucleotide was used to generate the SplintR-qPCR standard curve. Two miR-122 specific DNA probes were designed with flanking sequences to allow amplification using a forward and reverse PCR primers, miR-122 fp and miR-122 rp. The ligation substrate was prepared by hybridization of miR-122 oligonucleotide to two complementary DNA probes in the following amounts: 1 µl of miR-122 RNA oligos dilution (0.1 ng/µl to 0.1 fg/µl), 1 µl of 0.1 µM miR122 probe qA10, 1 µl of 0.1 µM miR122 probe qB12, 1 µl of 10× Splint R ligase buffer and 1 µl of *E. coli* rRNA (10 ng). Hybridization was achieved by first heating at 90–95°C for 5 min then slowly cooling to 51°C for 40 min in a PCR cycler. After annealing reactions were cooled to 37°C and 1 µl of Splint R ligase and 1 µl of 0.1 M DTT were added. The ligation reaction was incubated at 37°C for 2 h. For a negative control the miR-122 target was omitted.

The qPCR reaction for miR-122 detection contained the following components: 2 µl of the 7 µl of ligation reaction described above, 10 µl of One Taq Hot Start 2X PCR master mix (NEB), 2 µl of miR-122 double-quenched DNA probe (miR-122 dqp), 1 µl of miR122 forward PCR primer (miR-122 fp), 1 µl of miR122 reverse PCR primer (miR-122 rp) and 4 µl of H_2O. The qPCR reactions were carried out in triplicate in a 96-well plate. The plate was incubated in a qPCR thermocycler (BioRad CFX 96 Real-time system). The initial denaturation was 95°C for 3 min, followed by a two-step amplification: 95°C for 10 s and 55°C for 30 s for 40 cycles. The qPCR results were reported as the quantification cycle (Cq) that was determined by the BioRad cycler.

To determine the concentration of miR-122 in rat liver total RNA the same DNA probes used for the miR-122 standard curve were hybridized to different amounts of rat liver RNA. The hybridization reaction contained the following components:

1 µl of rat liver total RNA (1 ng/µl, 200 pg/µl, 40 pg/µl or 8 pg/µl), 1 µl of 0.1 µM miR122 probe qA10, 1 µl of 0.1 µM miR122 probe qB12, 1 µl of 10× Splint R ligase buffer and 1 µl of H_2O. Following heat denaturation the probes were hybridized at 51°C, ligated at 37°C and amplified by qPCR using the same protocol as the miR-122 standard curve. Since the qPCR reactions were done in triplicate the final amount of rat liver RNA in each qPCR assay was 300, 60, 12 and 2.4 pg (Figure 3B).

let-7 assay. The affect of temperature on the specificity of SplintR miRNA detection was investigated with members of the let-7 family. The hybridization and ligation reactions were done at the same time. The reactions with let-7b specific DNA probes contained: 1 µl of 0.1 µM probe qA5, 1 µl of 0.1 µM probe qB17, 1 µl (1 µg) of yeast RNA, 1 µl of 10× T4 DNA Ligase buffer, 4 µl of H_2O, 1 µl of 10 µM SplintR Ligase and 1 µl (10 fmol) of synthetic RNA for one of the 8 let-7 isoforms. The reactions were mixed well in a 0.2 ml PCR tube and incubated at 16, 25 or 37°C in a PCR cycler for 100 min. For the 37°C incubation the oligonucleotide mixture (9 µl) were pre-warmed at 37°C in the PCR cycler prior to the addition of SplintR Ligase (1 µl). This was to avoid ligation at the lower temperatures. The time and temperature for thermal cycling and the amount of DNA polymerase used in the assay are the same as those used for miR-122 detection described above. The double-quenched let-7 qPCR probe was used for miRNA detection in these studies.

Comparison of SplintR and TaqMan methods for miR-122 detection. A comparison of the sensitivity of TaqMan and SplintR for the detection of miR-122 was performed with three different amounts of rat liver total RNA, 1000, 200 and 40 pg. The protocol for qPCR amplification and detection of miR-122, using the double quenched probe, is described in the previous section.

The microRNA Reverse Transcription Kit for miR-122 and TaqMan Universal qPCR Master Mix were purchased from Life Technologies and performed according to the manufacturers instructions. This method uses a DNA hairpin as a primer for reverse transcriptase for synthesis of a cDNA copy of the miRNA. The miR-122 specific probe has a single non-fluorescent quencher, miR-122 sqp, at the 5′ end and a 3′ FAM probe. The qPCR conditions were the same as those used for the SplintR detection method.

Multiplexed detection of miRNAs from different tissues. The multiplexed detection of miRNAs was performed using a set of six DNA probes complementary to the following miRNAs: miR-17, miR-122, miR-34c, miR-350, miR-378a and miR-497. The hybridization mixture of DNA probes and miRNA contained: 2 μl (400–500 ng) of total RNA from different tissues, 2 μl of the mixture of six pairs of miRNA specific DNA probes, 1 μl of 10× Splint R ligase buffer and 3 μl of H_2O. For each pair of DNA probes the final concentration in the annealing mixture was 100 fmol/μl. The RNA and DNA probes were heated to 90–95°C for 5 min then slowly cooled to 37°C. Following hybridization 1 μl of Splint R ligase and 1 μl of 100 mM DTT were added and the ligation reaction was incubated at 37°C for 2 h.

The ligated probes were amplified by PCR prior to sequencing. The PCR reaction contained the following: 5 μl of the ligation reaction, 12.5 μl of LongAmp Taq 2X Master Mix (New England Biolabs), 1 μl of SR primer, 1 μl of index primer for multiplex (NEBNext Multiplex Small RNA Library Prep Set for Illumina) and 5.5 μl of H_2O in a total volume of 25 μl. Four index primers were used for the four individual miRNA libraries. The time and temperature for the PCR reactions were: an initial denaturation at 94°C for 30 s followed by 13 cycles at 94°C for 15 s, 62°C for 30 s and 70°C for 15 s. Final extension was at 70°C for 5 min. The amplified PCR products were gel purified and the quantity of DNA was measured with an Agilent 2100 Bioanalyzer. The four bar coded miRNA libraries were then mixed in an equal molar ratio and sequenced by MiSeq Illumina sequencer. The sequences of the miRNA targets and DNA probes are listed in Supplemental Material.

RESULTS AND DISCUSSION

We have developed a ligation based miRNA detection method. The first step involves hybridization and ligation of two miRNA-specific DNA oligonucleotides by SplintR ligase. The second step uses qPCR and a double-quenched FAM labeled DNA probe to specifically detect the amplified ligation product. A ligation based method allows more flexibility in probe design than existing methods and does not require the use of reverse transcriptase. SplintR Ligase was chosen because it is much more efficient in RNA splinted DNA ligation than either T4 DNA ligase or T4RNA ligase 2. To understand the advantages or limitations of a ligase based miRNA detection method we tested several parameters to determine the efficiency, sensitivity, specificity and substrate requirements for the SplintR ligation of DNA oligonucleotides probes in an DNA:miRNA hybrid. The SplintR method was also used for multiplexed detection of miRNAs from different tissues.

Two miRNAs, miR-122 and the let-7 family were chosen to validate the SplintR ligation method. MiR-122 is a very abundant liver specific miRNA. It is upregulated in patients with chronic hepatitis B virus (HBV) and hepatitis C virus. The miR-122 levels are associated with the level of HBV DNA (30). The let-7 miRNA family has eight members that differ by only a few nucleotides. Most of the variable nucleotides are located at the 3′ end of the miRNA. The let-7 family is studied because of their regulatory role as tumor suppressors. They are also important as regulators of embryonic development (31).

Length of DNA:RNA duplex requirement for SplintR ligation

The previous study that described the ability of SplintR Ligase to ligate DNA:RNA hybrid structures used DNA oligos (20 nt and 30 nt) complementary to a 50 nt RNA. Shorter RNA splints were also tested: an RNA splint of 20 nt could support ligation, but a 10 nt RNA splint did not allow ligation of complementary DNA oligos (28). To better define the minimal hybrid length required for ligation of DNA hybridized to a miRNA we designed 10 pairs of DNA probes that spanned miR-122 in two base increments (Figure 1A). These probes are named for the number of nucleotides complementary to the miRNA, for example probe A10 (10 complementary bases) was ligated to probe B12 (12 complementary bases). Probe A has a 3′ OH and is the acceptor for ligation, probe B has a 5′ phosphate and is the donor. Each probe also contained 21 or 22 nucleotide extensions to match PCR primers for qPCR amplification (see below). Gel electrophoresis was used to measure the extent of ligation of the FAM labeled probe A to unlabeled probe B with three different ligases (Figure 1B). Surprisingly, probe B required only four bases complementary to the 5′ end of miRNA-122 for ligation to probe A. However, the 3′ end of the miRNA required probe A to have 6 to 8 bases complementary to miR-122 for ligation. For comparison the same set of RNA:DNA hybrids was used to test the ability of T4 DNA Ligase or T4 RNA Ligase 2 to ligate DNA in an RNA:DNA hybrid (Figure 1B). Compared to SplintR hardly any ligation was observed with either of the T4 Ligases. Identical amounts of enzyme were used in all three reactions. These results demonstrate that SplintR Ligase requires only 4 to 6 complementary nucleotides in an RNA:DNA for ligation. Most of the nucleotides (10–12 nt) in the central region of the miRNA can be used as a junction for ligation of the hybridized DNA probes. This contrasts with the TaqMan method that uses a DNA hairpin primer with a short sequence complementary that is restricted to the 3′ end of the miRNA (22).

Kinetics of DNA:RNA duplex ligation

To obtain quantitative measurements of RNA splinted ligation, capillary electrophoresis (CE) was used to separate the unligated from ligated FAM labeled probe A. The CE traces of the ligation reaction show the unligated 32 nt substrate (S) and 65 nt ligated product (P) at three different reaction times (Figure 1C). Only FAM labeled probe A is detected, probe B is unlabeled. To obtain complete ligation the miRNA:DNA probe hybrid was designed to have limiting amounts of probe A. Both probe B (4×) and miR-122 (3×) were in excess of probe A. The percentage of ligation products versus time (Figure 1D) is shown for reactions using SplintR Ligase, T4 DNA Ligase and T4 RNA Ligase 2. There was a dramatic difference in ligation rates of the three enzymes. At the first time point, 5 min, SplintR Ligase had achieved complete ligation of the two probes. However, at 2 h T4 DNA Ligase and T4 RNA Ligase 2 have <10% ligated product. This means that under these conditions SplintR Ligase was over 200× more efficient in ligation of RNA splinted DNA probes than either of the T4 phage enzymes, consistent with previous reports (27,28).

Detection of miR-122 in rat liver total RNA using SplintR method

To test the utility of SplintR Ligase to detect miRNAs in a biological sample we developed a qPCR assay for miR-122. The SplintR ligation method has two steps: ligation of miRNA specific probes followed by qPCR detection. In qPCR reactions amplified DNA can be detected by binding to a dye, like SYBR Green that binds to all dsDNA (32), or by use of sequence specific DNA probe that contains a quencher and fluorophor. During amplification the 5′ to 3′ exonuclease activity of the DNA polymerase separates the quencher from the probe to generate a fluorescent signal (Figure 2). The miRNA-specific quenched probes are more specific than the SYBR Green method that binds to all dsDNA including primer dimers.

A standard curve was constructed in triplicate using tenfold serial dilutions of a synthetic miR-122 RNA oligonucleotide and 10ng/1μl *E. coli* rRNA to mimic conditions in a biological sample. The SplintR qPCR method could detect 30 ag of miR-122 (2500 copies of miR-122). The coefficient of determination, *R* squared, was 0.98 (Figure 3A). To test the sensitivity of the SplintR method using a biological sample we measured endogenous miR-122 in rat liver total RNA using the same protocol as was used for the standard curve. Since the assay was performed in triplicate the final concentration of rat liver total RNA in each qPCR reaction was 300, 60, 12 and 2.4 pg (Figure 3B). The amount of miR-122 in rat liver total RNA was calculated from the standard curve to be 62 pg/μg of rat liver RNA which agrees with the reported value of 60 pg/μg of total liver RNA (17).

A spike-in of miR-122 into RNA liver RNA was used as second method to measure miR-122 in the sample. Different amounts of synthetic miR-122 (from 30 to 0.03 pg) were added to a constant amount of rat liver RNA (60 pg). The miRNA was measured using the SplintR qPCR protocol described in the previous paragraph. As expected the 0.03 pg (30 fg) miR-122 spike-in was about 10–15-fold greater, (delta Cq 3.86), than the rat liver RNA sample that had no
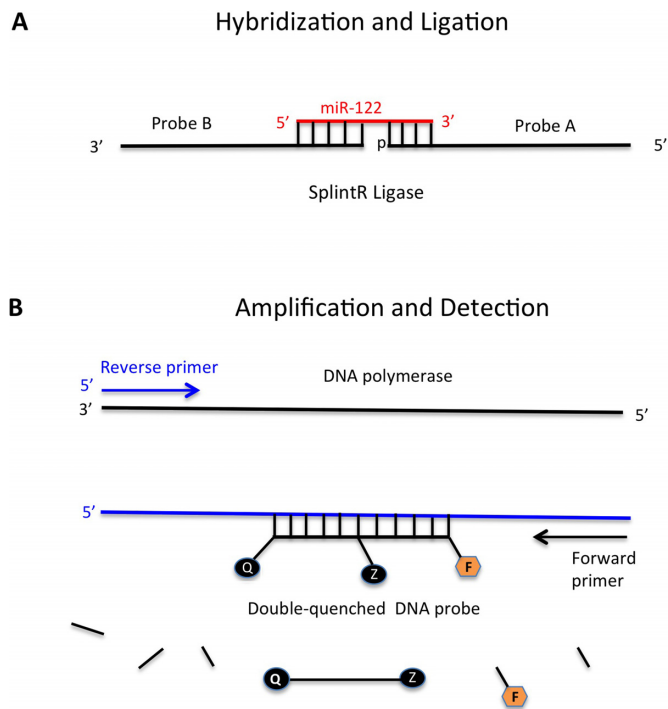

Figure 2. A schematic diagram of miRNA detection using SplintR ligation. The two step method first requires hybridization and ligation of two DNA probes complementary to a miRNA followed by amplification and detection. (**A**) The diagram shows miR-122 (red) hybridized to two DNA probes (black). Probe A has a 3′ OH and probe B a 5′ phosphate to allow ligation by Splint R ligase. The probes have extensions beyond the region complementary to the miRNA. The extensions are complementary to primers used for PCR amplification. (**B**) The ligated probes are amplified by a forward (black) and reverse (blue) DNA primers using a heat stable DNA polymerase. The PCR amplified product is detected by a double-quenched DNA probe that is complementary to miR-122. This probe, synthesized by IDT, has a 5′ terminal fluorescein dye, F, an internal ZEN quencher, Z, and a second quencher, Q, (Iowa Black Fluorescein quencher). During amplification the exonuclease activity of DNA polymerase separates the fluorophore from the quenchers generating a fluorescent signal. Because the primers and probes are in excess of the miRNA target the qPCR signal is proportional to the amount of miRNA in the reaction.

added oligo. Using a value for miR-122 in rat liver RNA of 60 pg/μg a liver RNA sample of 60 pg would have 3.6 fg of miR-122. The 30 fg miRNA spike in would also have 3.6 fg of endogenous miR-122 for a total of 33.6 fg which is ten fold greater than the endogenous amount. The protocol and qPCR tracings are presented in the supplementary data.

Comparison of miRNA detection by SplintR and TaqMan methods

The TaqMan method from Life Technologies is widely used for miRNA detection. The major difference between the SplintR qPCR and TaqMan method is the approach used for cDNA synthesis. The SplintR method uses ligation of complementary oligonucleotides to generate a DNA copy of the miRNA (Figure 2), while the TaqMan method uses a miRNA-specific DNA hairpin as a primer for cDNA synthesis by reverse transcriptase (22). We compared sensitivity of the two methods for their ability to detect miR-122 in dif-
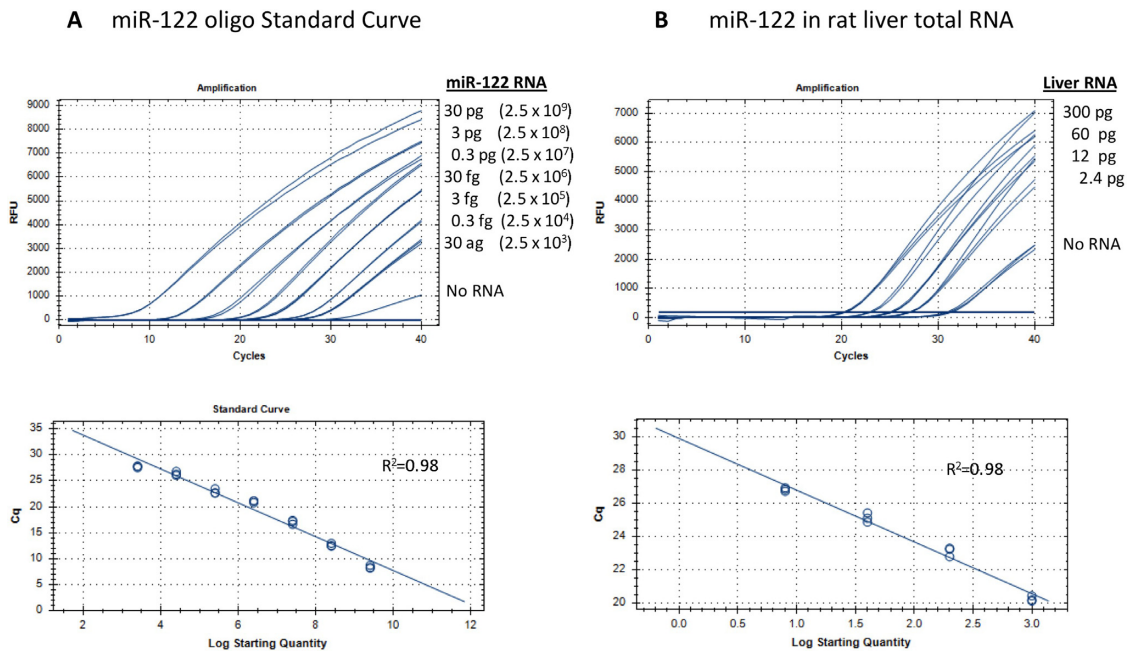
Figure 3. Detection of miR-122 in rat liver total RNA by SplintR ligation and qPCR amplification measured with double-quenched probe. The amount of miR-122 RNA and the number of molecules in each qPCR reaction is written at the right of the qPCR tracings. The qPCR assays were performed in triplicate using the double quenched miR-122 specific DNA probe shown in Figure 2. A negative control with no RNA was included in each assay. Details of the ligation and amplification are described in Materials and Methods. (**A**) The standard curve was generated with a 10-fold serial dilution of synthetic miR-122 RNA, from 30 pg to 30 ag (2.5×10^9 to 2.5×10^3 molecules). To mimic biological samples, *E. coli* ribosomal RNA was added to each of the reactions with diluted miR-122 RNAs. (**B**) Four different amount (1000–8 pg) of rat liver total RNA were used for miR-122 detection. Graphs of the Cq values versus the log of the miRNA or total RNA are shown at the bottom of each panel. They were used to calculate the amount of miR-122 in rat liver total RNA.

ferent amounts of rat liver total RNA (1, 0.2 and 0.04 ng). The SplintR method used a double-quenched FAM-labeled probe (29 nt) while the TaqMan method used a 5′ FAM fluorophor and a single 3′ non-fluorescent quencher (14 nt) from Life Technologies. For cDNA synthesis the TaqMan assay used the miR-122 specific TaqMan MicroRNA Reverse Transcription Kit and the TaqMan Universal PCR Master Mix. At all three RNA concentrations the SplintR method was more sensitive than the TaqMan method (Figure 4). For the 1 ng rat liver RNA the SplintR method was at least 40× more sensitive than the TaqMan assay. At the lowest level of liver RNA (0.04 ng) only the SplintR method could detect miR-122 (Figure 3).

There are several variables that could account for the differences in sensitivity of the two methods. One reason could be that SplintR ligation of DNA oligonucleotides is more efficient than reverse transcription for generation of the first strand cDNA. In the TaqMan method the DNA hairpin primer is restricted to the 3′ end of the miRNA. The SplintR method has flexibility in designing the location of miRNA-specific DNA oligonucleotides. The ligation junction can be at different positions in the RNA:DNA duplex so long as there is a 4 bp overlap of the miRNA with probe B and 6–8 bp overlap with probe A. These requirements allow 10–12 bases of the miRNA to be used as potential ligation junctions. The SplintR method also allows the use of the long double quenched qPCR probes. The major requirement for qPCR probe design is for the probe not to overlap with the PCR primers, which would inhibit amplification.

A second variable is the polymerase used for amplification. The SplintR method used One Taq Hot Start 2X PCR Master Mix while the other approach used the TaqMan Universal qPCR Master Mix. To determine if the polymerase was responsible for the difference in sensitivity we used either TaqMan Universal PCR Master Mix or the One Taq Hot Start for amplification of the SplintR ligation reaction. We found no differences in the sensitivity of miR-122 detection using either polymerase (data not shown). This makes it unlikely that efficiency of amplification was responsible for the differences we observed. The third variable is the miRNA specific DNA probes used in the qPCR assay. The ligation method used the double-quenched DNA probe with an internal ZEN quencher from IDT which results in a 1–2-fold decrease in the Cq value. The TaqMan probe for miR-122 is 14 nt long and has a single quencher, while the miR-122 specific probe for SplintR qPCR assay is 29 nt long. The greater length may enhance hybridization and improve sensitivity. It is likely that the 40-fold increase in sensitivity of the SplintR method is a combination of efficient ligation and improved sensitivity of the dual quenched probes.

Detection of let-7 isoforms using SplintR ligation

Members of miRNA gene families often have very similar sequences. For example there are eight described members of the let-7 family in humans that only differ by one or two nucleotides from other let-7 isoforms (31) (Figure 5A). We used the natural diversity of let-7 family to see if SplintR
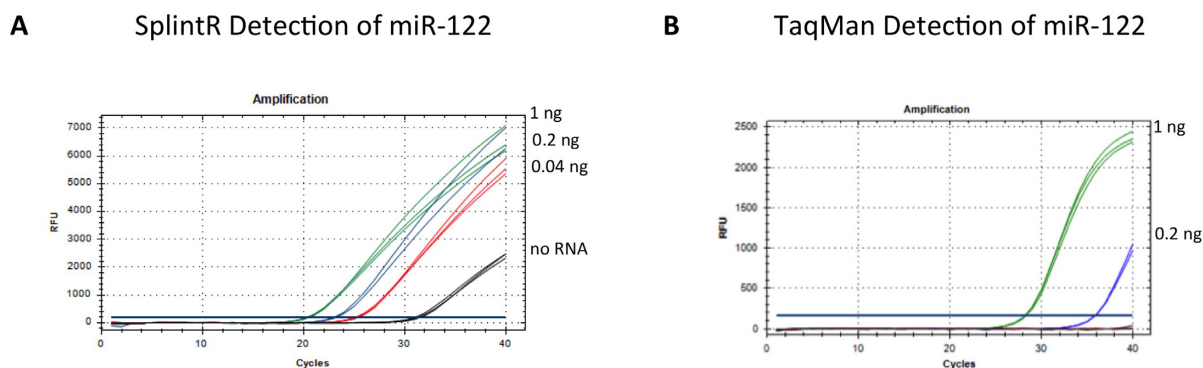
A   SplintR Detection of miR-122   **B**   TaqMan Detection of miR-122

Figure 4. Comparison of SplintR ligation method to TaqMan assay for detection of miR-122 in rat liver RNA. Three different concentrations of rat liver total RNA were used; 1 ng (green), 0.2 ng (blue), 0.04 ng/μ (red) and no rat liver RNA (black). (**A**) This panel shows the qPCR traces for the SplintR method described in Figure 2. The ligated probes were amplified by PCR and detected with a double quenched miR-122 specific DNA probe. (**B**) TaqMan detection method for miR-122 used a DNA hairpin complementary to the 3' end of the miRNA to prime cDNA synthesis with reverse transcriptase. The amplified product is detected by cleavage of a TaqMan probe specific for miR-122 (miR-122 sqp). The sequences of the probes and protocols for amplification and detection are described in Materials and Methods.

ligation could identify specific let-7 miRNAs We designed three pairs of DNA probes for the specific detection of let-7b, let-7c or let-7g. The specific probes are fully complementary only to their target sequences. The differences between the specific let-7 isoform and other members of the let-7 family are highlighted in red (Figure 5A). The ligation junction of the DNA probes is shown as a vertical line on the let-7 sequences.

In separate reactions let-7 isoform-specific A and B probes were hybridized and ligated in the presence of each of the eight members of the let-7 family. Negative controls include the absence of either probe A (−A), probe B (−B), SplintR Ligase (−R) or the stacking DNA oligo complementary to probe A (-S). For all three let-7 probe pairs, ligation was only observed when the correct let-7 isoform-specific probes were used (Figure 5B). A single nucleotide mismatch to the probes abolished ligation.

A stacking DNA oligonucleotide (stacking oligo) was used to enhance ligation for reactions that have a short overlap between the miRNA and probe A. The stacking oligo was complementary to probe A and is adjacent to the 3' end of the miRNA, thus generating a dsDNA region beyond the 3' end of the miRNA. The stacking oligo lacks a 5' phosphate and therefore cannot be ligated to the 3' end of the miRNA. The stacking oligos used in the detection of let-7b and let-7c, only had a 5 bp and 4 bp overlap between the miRNA and probe A (Figure 5B). Under these conditions, removal of the stacking oligo resulted in no ligation (−S). The smearing of the unligated probe A, at the bottom of the gel, was caused by hybridization of FAM labeled probe A to the stacking oligo. This was not observed in the controls (−S) that lacked the stacking oligo. The let-7g probe set, which had a longer six base overlap with the miRNA, did not require a stacking oligo for efficient ligation.

The stacking oligo may enhance ligation by creating a region of dsDNA at the 3' end of the miRNA that enhances binding of SplintR Ligase, this was in agreement with data shown in Figure 1B. The stacking oligo may also enhance the stability of the RNA:DNA hybrid by base stacking interaction between the 3' terminal base on the miRNA and

the adjacent 5' base of the stacking oligo. For dsDNA base stacking has been calculated to add ~1 kcal/mol to the stability of the duplex (33). A second possibility is that the stacking oligo creates a region of double stranded DNA that enhances ligase binding.

The let-7 study demonstrates that SplintR Ligase can discriminate single base mismatches on either side of the ligation junction. For example, there was only a single base difference between let-7b and let-7c, a G to A transition at the sixth base from the 3' end of the miRNA. This nucleotide difference is located on the donor (5'p) side of the ligation junction. A specific let-7b probe B was designed with a 5' terminal C that is complementary to the G in the miRNA. As seen in the upper panel of Figure 5A ligation was only observed for the correct match between the probes for let-7b and the correct let-7b miRNA. No ligation was observed to the other seven let-7 miRNAs. The SplintR Ligase can also discriminate between mismatches at the acceptor (3' OH) side of the ligation junction. Let-7c had a G at the fourth base from the end of the miRNA. However, five of the other let-7 miRNAs, which did not ligate to the probe, had an A at this position (Figure 5B, middle panel). This means that the ligase discriminates between the incorrect rA:dC mismatch and the correct rG:dC match at the 3' terminus of probe A.

Isoform specific detection was achieved even when the mismatched base was one base from the ligation junction. The ligation junction for let-7g is GU | AC while four other let-7 variants have GU | AU, where the vertical line indicates the ligation junction. There are two factors that determine the specificity of ligation: the ability of SplintR Ligase to selectively ligate correctly paired nucleotides and the stability of the RNA:DNA hybrid. It is likely that the specificity of the let-7g probe was due to both the selective hybridization of probe A to the miRNA and the specificity of the ligase for correct base pairing. When a stacking oligo complementary to probe A was included in the let-7g assay the ligation was less specific (data not shown). This observation suggests increasing the stability of probe A by inclusion of a stacking oligo reduces specificity of ligation at the $N-1$ position.
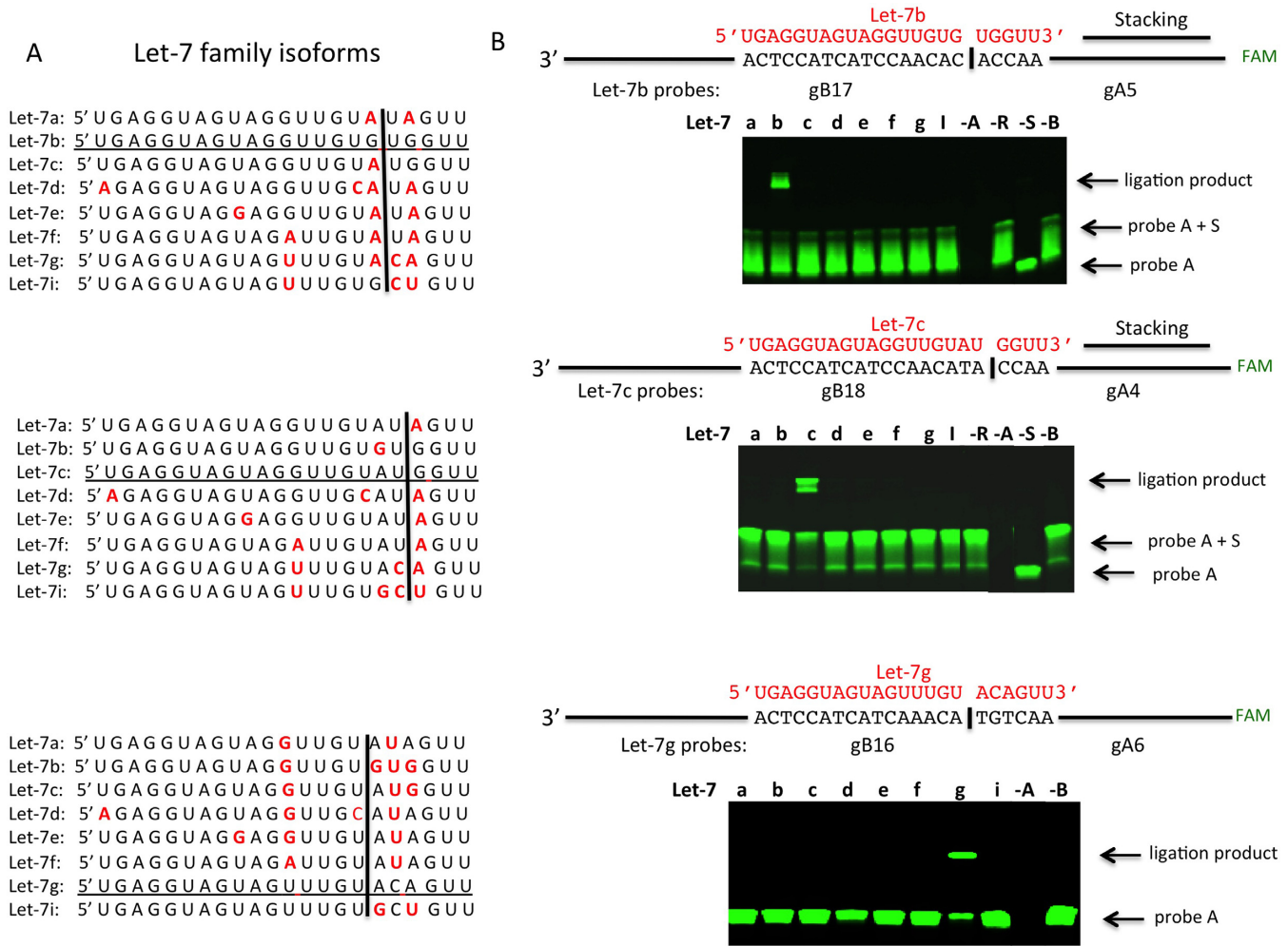
Figure 5. Specific detection of let-7 isoforms by SplintR ligation. (**A**) The aligned sequences of eight members of the let-7 family are shown. The sequence of the specific let-7 isoform to be detected is underlined in black. Nucleotides that do not match that isoform are shown in red. A vertical black line marks the ligation junction for the two probes. (**B**) Gel analysis of ligation reactions. Specific sets of DNA probes were individually hybridized to all eight let-7 miRNAs. A stacking DNA oligonucleotide, which is complementary to probe A, was included for the let-7b and let-7c isoforms to enhance the annealing and ligation. After ligation the products were seperated on a TBE urea polyacrylamide gel. The FAM labeled probe A was visualized using UV light. Negative controls include: no probe A (−A), no SplintR Ligase (−R), no stacking oligo (−S) and no probe B (−B). The oligonucleotides used in each ligation are shown above the gel. The miRNA is red, probe A, probe B and stacking oligo are black. Hybridization of the stacking oligo to probe gA5 and gA4 results in retarded mobility on the gel. In the absence of the stacking oligo (−S) a single band, probe A, is observed. The location of the ligation product, probe A and probe A hybridized to the stacking oligo are marked by arrows at the right of the gel.

Temperature and probe length alter ligation efficiency

Hybridization temperature is an important factor in the specificity of the SplintR Ligase detection method. To test this variable, let-7b specific DNA probes were hybridized, in separate reactions, to all eight let-7 miRNAs. The hybridization and ligation reactions were performed at three different temperatures: 16, 25 and 37°C. The let-7b-specific probe A has only five nucleotides complementary to the miRNA (Figure 6A). With this short overlap it was anticipated that specificity and maybe the sensitivity of the reaction would be temperature dependent. The hybridization and ligation reactions were done simultaneously. Each ligation reaction included a 15 000-fold excess (w/w) of non-specific yeast RNA compared to the miRNA target. The exogenous RNA was added to mimic biological conditions where miRNA often makes up <0.01% of the total cellular RNA.

The specific detection of let-7b was found to be very dependent upon the hybridization and ligation temperatures (Figure 6B). When the ligation reaction was performed at 16°C there was only a slight preference for detection of let-7b over the other let-7 isoforms. Most of the let-7 miRNAs differ by <4 Cq values from let-7b. The specificity for let-7b improved when the hybridization temperature was increased to 25°C, however, at 37°C there was a dramatic increase in specificity for let-7b detection compared to the other let-7 isoforms. Even after 40 cycles there was very little signal observed for let-7 isoforms other than let-7b. Surprisingly, improved specificity caused by increasing the hybridization temperature is not accompanied by a decrease in sensitivity. For all three temperatures the Cq value for let-7b is 18–20 cycles. Very specific ligation was achieved at 37°C, a temperature significantly higher than the calculated
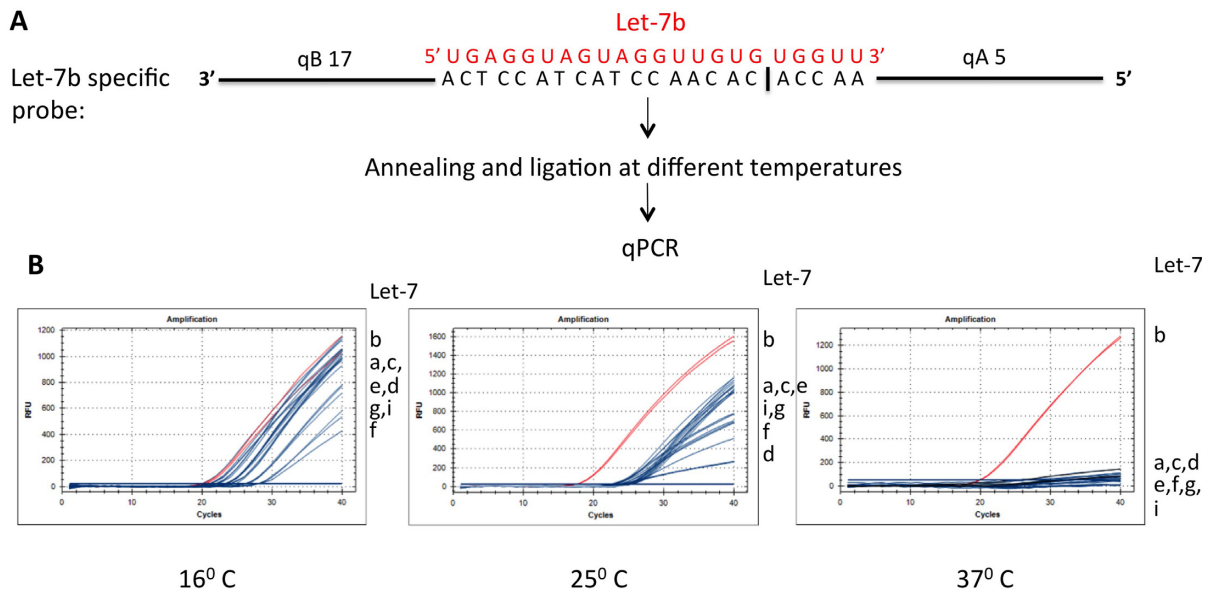
Figure 6. Ligation temperature enhances the specificity of let-7b detection. Probe qA5 and probe qB17 were hybridized to a mixture of eight different isoforms of let-7 and ligated with SplintR ligase at different temperatures. (**A**) The sequences of let-7b, in red, and the probes qA5 and qB17 complementary to let-7b are shown in black. (**B**) The annealing and ligation reactions were performed at 16, 25 and 37°C followed by qPCR amplification and detection with a let-7 specific probe. The qPCR traces for three different ligation temperatures are shown. The amplified DNA was detected with a double quenched let-7 DNA probe (let-7 dqp). The qPCR traces for the non-complementary let-7 isoforms, shown in blue, are noted by a single letter at the right of each graph. The correct let-7b tracings are marked in red. The reactions for each isoform were done in triplicate with the qPCR protocol described in Material and Methods.

Tm of 14°C for a 5 bp RNA:DNA hybrid. Probe A forms a short 5 bp duplex with the miRNA, however, probe B forms a 17 bp hybrid with the miRNA. The calculate T_m for probe B and the miRNA was 50°C (34). This suggests that the limiting factor for miRNA detection was hybridization and ligation of probe A to the more stable miRNA:probe B complex. The very high specificity of the reaction at 37°C is likely due to hybridization of probe A to only the complementary let-7 isoform. There is significant discrimination against other let-7 splints that differ by a single nucleotide (Figure 5A). Though the correct hybrid may be transient in nature at elevated temperatures it was sufficient for SplintR ligation. The rapid kinetics of SlintR ligation compared to other ligases (Figure 1D) are an advantage in ligation of short RNA:DNA hybrids.

The length of the hybrid between the miRNA and DNA probe was also an important factor for ligation. Three probes with different lengths of complementarity to the miRNA, let-7c (4 bp), let-7-b (5 bp) and let-7g (6 bp), were used for SplintR ligation followed by qPCR detection. The ligation product was amplified by PCR and detected using the dual quenched let-7 qPCR probe described above. Hybridization and ligation was at 37°C. As anticipated the longest hybrid between the probe and miRNA had the most efficient ligation. An increase in efficiency of ligation was measured by a decrease in Cq value required to detect the same amount of miRNA. When the number of nucleotides on probe A complementary to miR-122 was increased from 4 to 5 there was a 21-fold increase in sensitivity. When the length of hybrid was increased to 6 bp the sensitivity of the assay increases an additional 130-fold (Table 1).

Multiplexed detection of miRNAs using SplintR ligase

Libraries of synthetic miRNAs were used for a proof of principal study to detect, by SplintR ligation, multiple miRNAs in a single reaction. Six libraries were constructed using six or eight pairs of miRNA-specific DNA probes hybridized to a panel of 18 miRNA targets. Hybridized probes were ligated by SplintR, amplified and then sequenced on an Illumina MiSeq machine. The number of reads was used to determine the abundance of the miRNAs and the specificity of the ligase. Negative controls were included to test the specificity of ligation. Some libraries lacked one of the miRNA targets while others did not have one pair of DNA probes (Supplementary data Table S1). No false positives were observed, only specific miRNAs were detected that had both a target miRNA and the complementary DNA probes in the library. The experiment was designed to include the synthetic miRNA targets in equal amounts. The results show that the number of reads was within 2–3-fold of the expected value. For example if 6 miRNAs present in equal molar amounts each miRNA target had 5–20% of the total reads (expected value about 17%). The one exception was miR-411 which only had 0.5% of the total reads. This lower than expected value may be due to a six complementary bases near the end of miR-411 probe A that could result in dimer formation (Supplementary data Table S2). In designing probes for multiplexed detection care must be taken to avoid complementary sequences between either probes or PCR primers. Complementarity between the 3′ end of miR-411 probe A may allow primer dimers to be generated by DNA polymerase during amplification. But this is a specific issue that can be addressed by changes in primer design and by optimization of hybridization protocols. The SplintR en-

Table 1. Extent of complementarity of DNA probes to miRNA alters SplintR ligation

miRNA	Cq Value	Delta Cq	Fold Increase	miRNA:probe A overlap
let-7c	24.6 ± 0.1	0	1×	5′.... GGUU 3′ CCAA…..
let-7b	20.2 ± 0.6	-4.4	21×	5′…. UGGUU 3′ ACCAA…..
let-7g	13.2 ± 0.2	-11.4	2700×	5′…. ACAGUU 3′ TGTCAA….

Three pairs of let-7 DNA probes (A and B) with 4, 5 and 6 base pair complementary to their homologous miRNAs were used for SplintR ligation. The probe pairs used were qA4:qB18 for let-7c, qA5:qB17 for let-7b and qA6:qB16 for let-7g. The annealing and ligation reactions were performed for 1 h at 37°C. The same double quenched let-7 specific DNA probe was used for qPCR detection of all three let-7 isoforms. All three let-7 isoforms end in GUU so the probe was designed to be complementary to these last three nucleotides. The reactions were done in triplicate with the conditions described in Materials and Methods. The average Cq value for the three qPCR reactions and their standard deviation is shown. The fold increase in sensitivity is calculated from the delta Cq values assuming a doubling at each PCR cycle.

zyme allows flexibility in designing the location of ligation junctions for the two probes. Results and methods for the synthetic miRNA multiplexed libraries are described in the Supplementary data.

The multiplexed miRNA detection method was next tested on total RNA from rat liver, mouse kidney, rat testis and human brain. Probes were chosen for six different miR-NAs (miR-17, miR-122, miR-34c, miR-350, miR-378a and miR-497) that represent a range of expression levels. The RNAs were hybridized to DNA probes, ligated, amplified and sequenced as described in material and methods. The results (Table 2) show tissue-specific expression patterns for miRNAs. As expected miR-122 was the major miRNA in liver, representing 83% of all the reads. The other five miR-NAs were at least 200× lower. In kidney miR-17 (29%) and miR-378a (18%) were the two most abundant miRNAs. For rat testis miR-34c (79%) was the most abundant and in human brain miR-17 (47%) was the major sequence. The values obtain from ligation are not absolute numbers, they represent only the relative number of reads compared to other miRNAs in the pool.

Quantitative PCR was used to make a direct comparison of miR-122 in the different RNA samples. The assay used SplintR ligation and qPCR amplification with a miR-122 specific double-quenched probe. Six different RNA sample were used: kidney, testis, brain, liver, HeLa and Jurket cell. The value for miR-122 in liver was arbitrarily chosen as 100% and the relative percentage of miR-122 in the other samples was calculated from the delta Cq values. Mouse kidney had 1.5% as much miR-122 as rat liver. All of the other samples were within two fold of the negative control that had no RNA (Figure 7). The multiplexed detection of miRNAs using NextGen sequencing has the following reads for miR-122: rat liver 83% of reads, mouse kidney 3.6%, rat testis 0.07% and human brain 0.06% (Table 2). It is likely that the very low levels of miR-122 in brain (0.06%) and kidney (0.07%) represent the background of the ligation assay. This was confirmed by sequencing of the small RNAs from brain, kidney and testis using the NEB-Next small RNA prep kit. The rat testis RNA had only 2 miR-122 sequences per million reads, human brain had 1 read, while the mouse kidney RNA had 530 reads/million (D. Rodriguez, personal communication).

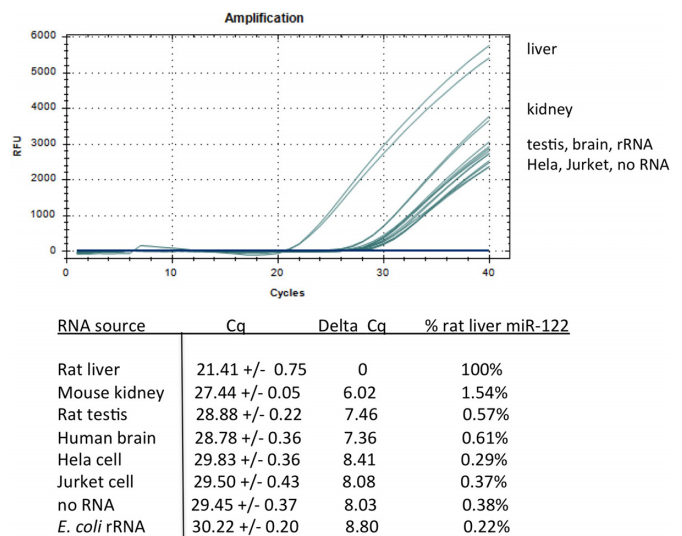MicroRNAs are attractive candidates for biomarkers. They can be detected in biological fluids, have tissue specific

RNA source	Cq	Delta Cq	% rat liver miR-122
Rat liver	21.41 +/- 0.75	0	100%
Mouse kidney	27.44 +/- 0.05	6.02	1.54%
Rat testis	28.88 +/- 0.22	7.46	0.57%
Human brain	28.78 +/- 0.36	7.36	0.61%
Hela cell	29.83 +/- 0.36	8.41	0.29%
Jurket cell	29.50 +/- 0.43	8.08	0.37%
no RNA	29.45 +/- 0.37	8.03	0.38%
E. coli rRNA	30.22 +/- 0.20	8.80	0.22%

Figure 7. Detection of miR-122 in different RNA samples by the SplintR qPCR. The relative amount of miR-122 in eight different RNA samples was measured by SplintR ligation followed by qPCR. The protocol was the same one used for the miR-122 standard curve. The qPCR tracings are at the top of the figure with the origin of the RNA samples listed at the right. Below the curves is the table listing the average Cq values and standard deviation. Each reaction was done in triplicate. The delta Cq value calculated by subtracting the rat liver Cq value of 21.41 from each of the other samples. The delta Cq was then used to calculated the relative amount of miR-122 compared to rat liver. For example, the kidney RNA sample required 6 additional PCR cycles, $2^6 = 64$, to detect miR-122. This means kidney RNA has 1/64th or 1.5% as much miR-122 as rat liver. The background with no RNA had Cq values of 29–30. All RNA samples except kidney and rat were within two fold of background.

expression and often correlate with the clinical and biological characteristics of tumors (5). MicroRNAs profiles have been described for a variety of cancers including, pancreatic, colorectal, osteosarcomas and clear cell renal cell (35). Accurate diagnosis requires monitoring changes in multiple miRNAs. Our proof of concept study demonstrates that SplintR qPCR is a sensitive and specific method for miRNA detection in biological samples. This method can also detect multiple miRNAs when coupled with NextGen sequencing. There are a variety of methods for detection of the SplintR ligated probes besides qPCR and DNA sequencing. For example the SplintR ligated DNA can be detected by microarrays (17), hybridization to multiplexed bead based suspen-

Table 2. Tissue specific miRNA expression determined by sequencing of multiplexed SplintR ligation products

miRNA	Kidney mouse	Liver rat	Testis rat	Brain human
miR-17	29.0%	0.15%	5.7%	47.7%
miR-122	3.6%	83.4%	0.07%	0.06%
miR-34c	1.0%	0.003%	79.1%	2.0%
miR-350	1.3%	0.01%	0.3%	0.005%
miR-378a	18.2%	0.2%	0.2%	1.2%
miR-497	7.3%	0.07%	0.5%	15.6%

A mixture of six pairs of DNA probes specific for miR-17, miR-122, miR-34c, miR-350, miR-378a and miR-497 were hybridized to total RNA from four different tissues and ligated by SplintR ligase. The ligation products were then amplified by PCR and sequenced on a MiSeq Illumina machine. The reaction conditions are described in Materials and Methods. The Table lists the % of total reads that match the six miRNAs from the four different tissues.

sion arrays (36) or multiple miRNAs can be detected by using different length PCR primers followed by gel electrophoresis (24). This initial report on SplintR is focused on the properties of the ligase and DNA:RNA substrates required for miRNA detection.

Additional research will be required to optimize detection of specific miRNAs and explore the use of SplintR ligase to detect miRNAs in clinical samples.

CONCLUSION

We have exploited the superior ability of SplintR Ligase to join DNA probes annealed to an RNA target to develop a sensitive and specific miRNA detection assay. This two-step method involves an initial ligation of two DNA oligonucleotide probes hybridized to a miRNA followed by qPCR amplification and detection with a double-quenched fluorophore probe. Recent reports have used T4 RNA Ligase 2 instead of SplintR Ligase to detect mRNA and alternative mRNA splice variants (37,38). However, T4 RNA Ligase 2 is not efficient in ligation of DNA oligonucleotides using an RNA splint. DNA probes that contain 3′ terminal ribonucleotides are required for efficient ligation. Our studies on ligation of DNA oligonucleotide hybridized to a miRNA (Figure 1C) show SplintR Ligase is 100× faster than either T4 DNA ligase or T4 RNA ligase 2. Complete ligation of DNA oligonucleotides was achieved by SplintR ligase in 5 minutes while T4 DNA ligase and T4 RNA ligase 2 achieved only 10% ligation after two hours (Figure 1D). We believe that the SplintR methodology will allow the development of assays with greater sensitivity for the detection of low amounts of target RNAs than either of the T4 enzymes. The improved sensitivity of the method allows miRNA detection in small quantities of starting material. The detection of miR-122 in 2.4 pg of rat liver RNA (Figure 3B) is less than the amount of total RNA in a single cell.

In designing miRNA specific probes an important property is the length of complementarity between the DNA probes and miRNA. We used a series of DNA probe pairs to scan the length of the miRNA to determine the minimum overlap required for ligation. Probe B, which contains the 5′p donor, requires only a 4 bp hybrid with the miRNA, however, probe A, which is the 3′ OH acceptor requires 6–8 bp complementary sequence for ligation. This means that when designing miRNA-specific DNA probes the majority of the internal miRNA sequences can be used for ligation junction. Probes were designed that could selectively detect individual members of the let-7 family that

differ by only single nucleotides, with the highest specificities observed when the ligations were performed at 37°C. Higher temperature increases the stringency of hybridization and improves specificity without sacrificing sensitivity. When coupled with qPCR the SplintR ligation method is very sensitive, it was able to detect a few thousand copies of miR-122 in a biological sample. The SplintR detection method, when coupled to NextGen sequencing was able to detect multiple miRNAs in a single reaction. These properties make the SplintR ligase a very useful enzyme for RNA detection.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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