Amplified microRNA detection by templated chemistry

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ABSTRACT

MicroRNAs (miRNAs) are a class of RNAs that play important regulatory roles in the cell. The detection of microRNA has attracted significant interest recently, as abnormal miRNA expression has been linked to cancer and other diseases. Here, we present a straightforward method for isothermal amplified detection of miRNA that involves two separate nucleic acid-templated chemistry steps. The miRNA first templates the cyclization of an oligodeoxynucleotide from a linear precursor containing a 5'-iodide and a 3'-phosphorothioate. The sequence is amplified through rolling circle amplification with φ29 DNA polymerase and then detected via a second amplification using fluorogenic templated probes. Tests showed that the cyclization proceeds in ~50% yield over 24 h and is compatible with the conditions required for rolling circle polymerization, unlike enzymatic ligations which required non-compatible buffer conditions. The polymerization yielded 188-fold amplification, and separate experiments showed ~15-fold signal amplification from the templated fluorogenic probes. When all components are combined, results show miRNA detection down to 200 pM in solution, and correlation of the detected signal with the initial concentration of miRNA. The doubly templated double-amplification method demonstrates a new approach to detection of rolling circle products and significant advantages in ease of operation for miRNA detection.

INTRODUCTION

The sensitive detection of RNAs is crucial in the analysis of gene expression and of RNA-dependent gene regulation. However, because RNAs vary widely in size, structure, localization and number within organisms, the development of specific RNA detection techniques remains a challenge. Methodologies are needed for detection both in whole tissues and organisms, in individual cells and in extracellular fluids. The biologically active RNAs themselves can be many kilobases in length or only a few nucleotides, and can be single-stranded, double-stranded or folded into widely varied structures with varied thermodynamics. Thus, methods that are to be useful need to address these issues either separately or together.

Among the most important and challenging RNA targets for detection are microRNAs (miRNAs), which are small regulatory RNA molecules that modulate gene expression by interacting with the translational machinery. MiRNAs present a particular challenge in detection because of their limited size and high sequence homology. Mature miRNAs are only 19–23 nt in length, often differ from one another by a single nucleotide, and are highly variable in their expression levels in cells (1). These parameters put significant demands on standard RNA detection techniques such as reverse transcriptase–PCR, and require a higher level of sensitivity than a simple fluorogenic reaction can provide (2).

Despite such challenges, miRNA detection is a very important goal. It has been determined that >60% of all mammalian messenger RNA transcripts contain conserved miRNA target regions (3). miRNAs have been shown to play key roles in plants and animals in developmental timing, cell fate and cell death (4,5). Furthermore, research suggests that abnormal miRNA levels are closely linked to cancer, and may be useful in cancer detection, diagnosis and treatment (6–8). In particular, the let-7 family of miRNAs has been found to play a role in tumor suppression by modulating ras and other oncogenes (9–11). Let-7a, the miRNA target in this study, is specifically of interest because in contrast to other members of the let-7 family it may act in an oncogenic manner, having been shown to target caspase-3 mRNA (12,13).

One method studied recently for miRNA detection is that of rolling circle amplification (RCA), which is an isothermal amplification approach involving a small circular DNA as a polymerase substrate (14–16).
single-stranded circular DNA template, producing hundreds of concatenated single-stranded copies of the circle sequence per hour. This amplification can be linked to detection of RNA by using the RNA target as a splint to bring together two ends of a precircle, allowing cyclization to occur through enzymatic ligation. In addition, the RNA can behave as a primer for the polymerase (15,17,18).

The ligation of a circle precursor oligonucleotide in RCA-mediated RNA detection methods is typically carried out with T4 DNA ligase (14,16,19,20). However, this ligase is not optimal for ligation of DNA on an RNA template, and so careful optimization is necessary to achieve a satisfactory yield (19,20). Additionally, this ligase shows relatively poor sequence specificity on an RNA target (15). The requirement for a separate enzyme also likely prevents application in intact cells and tissues. Moreover, the need for two enzymes (a ligase and a polymerase) adds to the complexity and cost of such detection methods. We considered the possibility that a chemical ligation method might ameliorate some of these limitations. Chemical ligations can be highly selective to sequence variations, and since they require no added protein, might provide a strategy for future use in cells. Autoligation chemistries are particularly attractive for RNA detection, since they require no added reagents as they rely on chemical functional groups placed on the DNA itself. Previous work has shown that autocyclization of an oligonucleotide with a 5'-iodide and a 3'-phosphorothioate can proceed via templated nucleophilic substitution in up to 65% yield (21). We therefore chose to investigate this method as a potential alternative to enzymatic ligation.

Common RNA detection methods require not only an amplification strategy but also a means of producing a signal for detection. RCA products are single-stranded and thus can be detected by the use of complementary probes, leading to a sequence-specific signal (14,22,23). The drawback of such simple hybridization probes is that the amplification arises only from the rolling circle activity, and thus sensitivity can be an issue. Alternatively, a branched rolling circle strategy has been developed, whereby a second primer can be used to create double-stranded DNA with a higher degree of amplification; these products can then be detected using an intercalating dye (15,16,24). However, there is no sequence-specificity in such a detection method, and so contaminants could lead to non-specific signal. Moreover, multiplexing would be difficult given the non-specificity of the dye.

Once again, we turned to the possibility of using templated chemistry to address these problems in detection. Quenched Staudinger-triggered (Q-STAR) probes are sequence-specific fluorescence turn-on probes that have low background, high positive signal and high sequence specificity (25). These probes use the bioorthogonal Staudinger reaction to cleave a quencher from a fluorescently-labeled DNA probe strand, and have been shown to function both in vitro and in intact cells. Q-STAR probes have demonstrated up to 100-fold isothermal turnover per target, which might significantly enhance the signal generated from a rolling circle reaction. With these considerations in mind, we have devised an isothermal double amplification technique for miRNA detection consisting of (i) RNA template-dependent autoligation of a circle precursor; (ii) RCA using φ29 DNA polymerase and primed by the RNA sequence and (iii) fluorescence detection of the rolling circle products using Q-STAR probes (Figure 1). Here, we describe the development of the methods and probes and their performance in the detection of let-7a RNA.

**EXPERIMENTAL SECTION**

**Preparation of oligonucleotides**

All DNA oligonucleotides were prepared on a 1 μmol scale on an ABI 392 synthesizer using standard phosphoramidite chemistry. Columns, phosphoramidites and reagents were purchased from Glen Research. DNA strands were cleaved and deprotected overnight at 55°C in concentrated ammonium hydroxide and purified using PolyPak II columns (Glen Research). RNA oligonucleotides were synthesized by the Stanford Peptide and Nucleic Acid Facility and purified by 20% polyacrylamide gel electrophoresis. 5'-iodo-3'-thiophosphate CPG strands were prepared by using a 3'-phosphate CPG column (Glen Research) with a sulfurizing reagent (Glen Research; Sulfurizing Reagent II) at the first oxidation step (the thiophosphate group is shown as s- in sequences), and addition of 5'-iododeoxyribothymidine phosphoramidite (IT) at the 5'-end of the strand (21). These circle precursors...
were deprotected for 24 h at room temperature in concentrated ammonium hydroxide, filtered, lyophilized and used without further purification. 5-methyl-dC was incorporated in each of the binding arms of the circle pre-cursor to increase the melting temperature (26). The sequence was 5'-TACTACTATTTCTTC TGCAGACTCGACTATACACCS'-3' (binding arms underlined) and the splint sequence (let-7a) was 5'-UGAGGUAGUAGGUAGUAGU-3'.

Ligation

A 50-mer circle precursor DNA internally labeled at two positions with fluorescein-dT (FlT) was synthesized: 5'-TACTACCTATTTCTTC TGCAGACTCGACTATACACCS'-3'. For enzymatic ligation, the same sequence was used with a 3' hydroxyl group and a 5' phosphate added with 5' phosphorylation reagent (Glen Research). Autoligation reactions were carried out at 50 nM concentrations (unless otherwise stated) in 1 x φ29 DNA polymerase buffer [50 mM Tris–HCl, 10 mM MgCl2, 10 mM (NH4)2SO4, pH 7.5] with a total reaction volume of 100 µl at 30°C, or as given. After the specified reaction time, the reaction was lyopholized, then redissolved in 10 µl of 9:1 formamide: 10 x TBE buffer and run on a 10% denaturing polyacrylamide gel. The fluorescent label was visualized on a GE Typhoon 9410 gel scanner with a 488 nm laser and 520 nm emission filter.

Enzymatic ligations were carried out with 20 nM RNA splint and 20 nM fluorescently labeled precircle in 1 x T4 RNA ligase 2 buffer (50 mM Tris–HCl, 2 mM MgCl2, 1 mM DTT, 0.4 mM ATP, pH 7.5; New England Biolabs). Reactions proceeded for 6 h at room temperature, before being denatured at 65°C for 15 min. 0.2 U/µl T4 RNA ligase 2 or 40 U/µl T4 DNA ligase were used. After denaturation, reactions were lyopholized, then redissolved in 10 µl of 9:1 formamide: 10 x TBE buffer and run on a 10% denaturing polyacrylamide gel and visualized as described above.

To determine the effect of buffer on RCA, either 1 x T4 RNA ligase 2 buffer or 1 x φ29 DNA polymerase buffer [50 mM Tris–HCl, 10 mM MgCl2, 10 mM (NH4)2SO4, pH 7.5] was used. In a total volume of 60 µl were combined 2 pM circle, 200 nM primer, 1 mM dNTPs for 12 h at 30°C. Products were then denatured 15 min at 65°C. Reactions were analyzed by agarose gel electrophoresis. Samples were mixed 5:1 with 20% glycerol loading buffer, and 6 µl/well were loaded on an 8% agarose gel. The gel was run for 3 h at 50 V, then visualized by staining with SYBR gold.

Triphenylphosphine probes and Q-STAR probes

All reagents for phosphoramidite synthesis were obtained from Glen Research. Triphenylphosphine (TPP) probes were made as previously described (27), with the exception that the ethanol precipitation step was omitted and the solution was purified by HPLC directly after conjugation. For most experiments (as indicated), two 2'-O-Me monomers (menoN) were incorporated at the 3'-end to protect from exonuclease cleavage (22). Sequences were as follows: 20-mer TPP: 5'-TCATCATTTCTCTCGTG menoNmenoNAG TPP-3'; 9-mer TPP: 5'-TCGTG TGmenoNmenoNAG TPP-3'.

Q-STAR probes were synthesized as previously described (25), with two 2'-O-methyl RNA monomers in the second and third positions from the 3'-end of the strand. Sequences were as follows: 18-mer Q-STAR: 5'-Q-STAR ACFlTGGACTTTAACTmeoAR-3'; 10-mer Q-STAR: 5'-Q-STAR ACFlTGGACTTTAACT-3'. The identity of probes was confirmed by MALDI-TOF mass spectrometry (Supplementary Table S1).

Q-STAR stability

18-mer Q-STAR probes with and without 2'-O-Me bases at the 3'-end were prepared. The fluorescence of 1 µM Q-STAR in 1 x φ29 DNA polymerase buffer with or without 0.5 U/µl φ29 DNA polymerase was monitored in a Fluoroskan Ascent microplate reader for 6 h.

TPP stability

Stability was monitored both by fluorescence and HPLC. For fluorescence, 600 nM solutions of 20-mer TPP probe in 1 x φ29 DNA polymerase buffer with or without 4 mM DTT were incubated at 30°C for 0–7 h. After incubation, 200 nM of probe template and 200 nM of Q-STAR probe were added, and the fluorescence was monitored for 1 h. For HPLC, 1.2 µM solutions of TPP in 1 x φ29 DNA polymerase buffer with or without 4 mM DTT were incubated at 30°C and injections were made every 80 min. A gradient of 5–40% acetonitrile in 50 mM TEAA buffer pH 8.5 was used. The peaks corresponding to the ratio of oxidized and unoxidized TPP probes were integrated, and the relative ratio calculated.

Q-STAR probe standard

In order to test the probes and quantify RCA, a 50-mer template for the probes was purchased (Stanford Peptide and Nucleic Acid Facility): 5'-GTGTATAGTTAAAGT CCGAGTCTCGACCGAGAATGATGAGGTAGT-3'. This template was made up in a series of standard concentrations (5 nM to 1 µM) in 1 x φ29 DNA polymerase buffer (described above). Standards also included 1 mM dNTPs, 10 nM precircle and 0.25 U/µl of denatured φ29 DNA polymerase.

RCA and detection

A 10 x buffer for φ29 DNA polymerase was prepared or purchased [500 mM Tris–HCl, 100 mM MgCl2, 100 mM (NH4)2SO4, pH 7.5, with or without 40 mM dithiothreitol (DTT)]. Reactions contained a total volume of 50 µl with 1 x buffer, 1 mM dNTPs, 10 nM precircle, 0.25 U/µl φ29 DNA polymerase and the appropriate concentration of RNA (100 pM to 10 nM). Reactions were heated at 30°C for 24 h or as indicated, then denatured at 65°C for 15 min. Progress of rolling circle reactions was analyzed by 0.8% agarose gel electrophoresis using SYBR Gold staining. Q-STAR detection of products was carried out by adding 10 µl of reaction to a 384-well small volume
non-binding microplate (Greiner) with 400 nM Q-STAR probe and 1.2 μM TPP probe. A Fluoroskan Ascent microplate reader was used to monitor the fluorescence at 538 nm at 30°C over 8 h.

In some cases, it was desired to test RCA separately from ligation; in these cases, 2 pM preformed circle replaced the precircle, and 200 nM RNA were used. All other methods remained the same.

RESULTS
Probe cyclization by RNA-templated autoligation
The phosphorothioate-iodide circle ligation was investigated for efficacy at lower concentrations of precircle and template than had previously been reported (21). Products were observed by gel electrophoresis. At 50 nM concentrations, the reaction proceeded over 24 h to a total yield of 45% as measured by relative fluorescence (Figure 2). A ~100-mer apparent dimer was also present at significant (~20%) concentrations both in the presence and absence of RNA splint. The dimer concentration could be reduced in the presence of DTT, suggesting that it was due to disulfide formation rather than a substitution reaction (Supplementary Figure S1), but we ultimately chose not to use DTT in our reactions due to incompatibility with the TPP probe (see below). Further experiments showed that the ligation yield was not affected by the presence of φ29 DNA polymerase, suggesting that autocycling could be compatible with RCA for a single-tube reaction (Supplementary Figure S2).

We also compared autocycling to other ligation methods. In our hands, cyanogen bromide ligation (28) and EDCI-mediated circle closure (29) gave none of the desired circular product (data not shown). T4 DNA ligase and T4 RNA ligase 2 yielded circular product in the presence of RNA splint, but under buffer conditions that were not directly compatible with RCA (Supplementary Figure S3).

Stability of Q-STAR and TPP probes
Since Q-STAR probes had been used successfully under biological conditions in the literature (25), we did not anticipate problems in the conditions typically employed for RCA. However, initial attempts to monitor the production of rolling circle products as they were generated met with limited success using these fluorogenic probes. Control experiments revealed that unmodified Q-STAR probes yielded a signal in the presence of φ29 polymerase regardless of whether a target RNA or a TPP probe was present (Figure 3). Literature reports describe a 3′-exonuclease activity in this polymerase, and so we considered the possibility that full polymerase-mediated degradation of the probes to cut the fluorescein-labeled thymidine from the quencher at the extreme end might yield unwanted background signal. To address this, we incorporated two 2′-O-methyl nucleosides at the 3′-end of each of the probes (22). The modification succeeded in preventing the false signal: protected probes gave very little signal with the polymerase alone (Figure 3).

We also observed limitations in the stability of the TPP probes under the initial conditions studied. We found that reactions reached a plateau sooner than expected for the given amount of Q-STAR probe. Moreover, we observed that TPP probes were limiting, and addition of further equivalents increased the signal (data not shown). Further investigation led to the identification of DTT in the enzyme buffer supplied by the manufacturer as a significant source of the problem. Fluorescence experiments showed that incubation of the TPP probe alone in a buffer containing 4 mM DTT before adding the probe template and Q-STAR probes caused a significant decrease in fluorescence relative to adding the Q-STAR probe and template initially, as well as relative to incubation in a buffer without DTT (Figure 4). Additionally, HPLC experiments revealed that a significant portion of the TPP probe became oxidized after incubation with the DTT-containing buffer, but the oxidized portion of TPP remained relatively constant in the buffer in which DTT was absent (Supplementary Figure S4). We hypothesized that the oxidized form of DTT (the disulfide) was acting as an oxidant for the phosphine of the TPP probe, a reaction reported previously (30). Agarose gel quantification of rolling circle products showed that the φ29 DNA probes were compatible with RCA for a single-tube reaction (Supplementary Figure S2).

Figure 3. 2′-O-methyl nucleotides at the 3′-terminus protect probes from degradation. False signal from unprotected Q-STAR probes with φ29 DNA polymerase alone (red). Protected probes with two 2′-O-Me nucleosides at the 3′-end (blue); Q-STAR probes without polymerase (black). Experiments were carried out with 1 μM Q-STAR and 0.5 U/μl polymerase at 30°C.

![Figure 2](https://academic.oup.com/nar/article-abstract/40/9/e65/1135304)
polymerase enzyme performed comparably with and without the presence of DTT in the buffer (data not shown), so further experiments were carried out in a buffer without DTT, ameliorating the problem of insufficient reactivity of TPP probes.

Quantification of rolling circle products with Q-STAR probes

We hypothesized that it should be possible to quantify amplification of the target achieved through the rolling circle process; each cycle of replication around the circle by φ29 polymerase produces one copy of the original target sequence and one copy of the probe-binding region. Long (18–20-mer) Q-STAR probes were employed to prevent dissociation of the probes from the target sequence in order to have one fluorescence turn-on event correspond to one repeat unit in the RCA product. We tested the use of Q-STAR probes as reporters both simultaneous with the RCA step and after termination of RCA by thermal denaturation. We found that addition of the Q-STAR probes after termination of RCA yielded higher ultimate signals (Supplementary Figure S5), and so this approach was used to perform quantification studies.

In order to quantify amplification, a standard curve was constructed to correlate the concentration of probe binding sites to the fluorescence output (Supplementary Figure S6). A 50-mer linear DNA containing a site complementary to the probes was prepared, and the fluorescence signals generated from known concentrations of this template were used to create the standard curve. The linear regression equation from this data would allow determination of the concentration of probe binding sites in a sample from the fluorescence signal.

Once a standard curve had been created, it could be used to quantify products from our miRNA-templated ligation and RCA. Known concentrations of miRNA were incubated under ligation-RCA conditions for 24 h, at which point the probes were added. The fluorescent signal was recorded 200 min after addition of the probes and compared to that of the templated standards. Data showed that miRNA concentrations greater than 2 nM generated more than 400 nM of probe binding sites through ligation-RCA, and thus could not be quantified with the 400 nM of Q-STAR probe used. Concentrations of miRNA lower than 0.2 nM could not be distinguished from background. Thus, under this set of conditions the use of the long (non-turnover) Q-STAR probes gave a 10-fold sensitivity range for quantitative detection of miRNA (Figure 5). However, adjusting the probe concentration would change the range of concentrations of miRNA that can be quantitated.

Within the detection range of 0.2 nM–2 nM, it was possible to calculate the amplification generated through the rolling circle reaction. Using the 1 nM miRNA sample, 188 nM of probe binding sites were generated, corresponding to 188 copies of the circle or incorporation of 9400 bases per miRNA in 24 h.

Double amplification detection

Since Q-STAR probes have been shown to undergo turnover with DNA targets (25), we carried out studies to test whether the probes in the current application could yield a second level of amplification after RCA. With this in mind, we created shorter (9- and 10-mer) Q-STAR and TPP probes that were expected to bind more weakly, thus allowing them to dissociate from the template after reacting to generate a signal. This might then enable a given template site of the rolling circle multimer to catalyze multiple reactions. Using the turnover probes, we indeed saw enhanced signal as compared to the long probes, corresponding to a maximum turnover number of ~15 (Supplementary Figure S7). However, the enhanced amplification did not improve the detection limit as expected. For both the long and short probes, no signal above background was seen for samples containing 0.1 nM RNA or less, suggesting that RCA or autoligation failed to proceed at these low concentrations.

Since turnover with the shorter probes was possible, maximum signal could no longer be correlated linearly with concentration. We found that a more useful measure to relate to RNA concentration for the short probes was the initial rate of reaction as measured by the slope of the fluorescence curve. Several trials showed that initial rate correlated well with RNA concentration over the range of concentrations 0.2–10 nM (Figure 6).

Mismatch sensitivity

Autoligation via nucleophilic displacement of iodide was previously shown to have some sensitivity to single-nucleotide mismatches in the template strand, particularly toward mismatches in the center of the binding arm regions (21). Sequence selectivity is an important goal
in miRNA detection, as miRNA sequences within families often vary by one or two nucleotides. The ligation/RCA/Q-STAR method was tested for sensitivity toward let-7a against two closely related miRNA sequences, let-7d and let-7f. Let-7f places a mismatch close to the ligation site, while let-7d has a mismatch several bases from the ligation site and an additional mismatch at the 3’-end of the target (Table 1). The mismatch strands were incubated under the ligation-RCA conditions for 3–24 h, after which Q-STAR probes were added. The results showed no selectivity for let-7a over let-7d, but 5-fold or greater sensitivity against let-7f at incubation times up to 18 h (Figure 7). As an additional test, a random miRNA sequence was added to a let-7a sample in equal or greater concentration. This RNA had no effect on the ultimate Q-STAR signal, indicating no interference from a random sample (data not shown).

DISCUSSION

Our experiments show that an miRNA sequence can be successfully detected in a single-tube experiment involving two separate templated chemistry steps combined with RCA. Previous studies have shown that target-primed RCA is well-suited to the small size of miRNAs (2,15,16). However, the earlier studies required a separate ligase enzyme to carry out the cyclization reaction. The current studies are the first to test a chemical autoligation for miRNA detection. Our data show that this templated reaction is completely compatible with RCA, allowing ligation and amplification to occur concurrently. The autoligation method presents several advantages over enzymatic ligations. First, fewer components are involved in the reaction, reducing

Table 1. Sequences tested for mismatch sensitivity

| let-7a    | TGA GGT AGT AGG TTG TAT AGT T |
| let-7d    | AGA GGT AGT AGG TTG CAT AGT T |
| let-7f    | TGA GGT AGT AGA TTG CAT AGT T |

Figure 5. Quantification of rolling circle copies from initial miRNA concentration. Autoligation and RCA were carried out simultaneously with 0 nM, 0.1 nM, 0.2 nM, 0.5 nM, 1 nM or 2 nM of RNA target for 24 h. After enzyme denaturation, 400 nM Q-STAR and 1.2 μM TPP were added. (A) Fluorescence traces with long QSTAR probes. (B) For the samples that gave a signal above background, the fluorescein signal was determined after 200 min and compared to the standard curve to calculate the concentration of circle copies. Error bars represent the standard deviation from six trials.

Figure 6. Correlation between initial rate for turn-on of short Q-STAR probes and initial concentration of miRNA. Ligation and RCA were carried out simultaneously with 0.005 nM, 0.02 nM, 0.1 nM, 0.2 nM, 0.5 nM, 1 nM, 2 nM, 5 nM or 10 nM of RNA target for 24 h. After enzyme denaturation, 400 nM Q-STAR and 1.2 μM TPP were added. The initial rate was taken as the average slope from 5–20 min after the addition of probes. Error bars represent the standard deviation from 5 trials.
complexity and cost. Second, only a single denaturation step is needed since there is only one enzymatic step. Third, the difficulties presented by optimizing two different enzymatic reactions are avoided. Finally, the ligation works equally well on an RNA and a DNA template (data not shown). A shortcoming of this method is the lack of selectivity for single nucleotide polymorphisms distant from the ligation site. However, previous work showed that autoligation could be sensitive to mismatches away from the ligation site (21). With optimization of melting temperatures and the site of ligation, higher selectivity should be achievable. T4 DNA ligase similarly shows mismatch sensitivity only within a few bases of the ligation site (15).

The results have shown that Q-STAR probes are successful as reporters for detection of miRNA through templated reaction on rolling circle products. A suboptimal fluorescence signal from concurrent detection and RCA necessitated a two-step process of ligation and RCA followed by denaturation of the enzyme and addition of the probes for fluorescence detection. In spite of this, Q-STAR probes present a unique advantage in being sequence-specific and being able to amplify the fluorescent signal through turnover. A turnover strategy for the detection of RCA products using molecular beacons has been reported, but it requires an additional enzymatic step to induce turnover and the sequence of the beacons is limited by the requirement for an endonuclease restriction site (23). Finally, Staudinger-based fluorescent probes have been described recently for the direct detection of miRNA in cells (31). However, the limit of detection has not been investigated and is potentially limited as there is no amplification analogous to RCA in the current study.

While robust methods for miRNA detection exist with commercially-available probes, such as reverse-transcriptase PCR and Northern blotting, the drawbacks of these methods have prompted continued interest in developing new miRNA detection techniques. Direct in situ detection avoids modification of miRNA prior to detection as required by PCR, can be highly sensitive, and can give localization information (31–33). However, such methods generally require stringent washing to rid samples of unhybridized probes, and sensitivity remains an issue. Many groups have been working toward microarray miRNA profiling techniques, which have the capability to provide information about multiple miRNAs and are sensitive down to femtomolar concentrations (34–36). While microarrays are advantageous for many applications, they often require optimization of hybridization probe lengths and lack the simplicity of solution-based detection. Detection of miRNAs in solution holds the most promise for diagnostics where levels of only a few miRNAs are of interest, and to that end, several techniques have been reported in the literature, with sensitivities in the femtomolar to picomolar range (15,16,37,38). The sensitivity of our technique, 200 pM, is comparable to that of northern blot miRNA detection, and would require micrograms of total cellular RNA (39). Although our proposed method lacks the sensitivity and selectivity of some reported techniques at this stage, its simplicity and the potential for use of 2–3 different colors for different miRNAs offer advantages, especially for RNAs that exist in reasonably high copy number. Additionally, with further optimization we expect this method may be able to achieve greater sensitivity, as the theoretical amplification from the combination of RCA and Q-STAR may be as high as ~10⁶. With our current protocol, RCA has been identified as the limiting factor in the detection limit. We postulate that this is due to the strong exonuclease activity of φ29 DNA polymerase, which can cleave ssRNA as well as DNA (40). When very low concentrations of the target are present, degradation of the precircle, the target itself, or the product may dominate over polymerization. Optimization of the enzyme concentration at low substrate concentrations or use of φ29 mutants or of other nuclease-deficient polymerases may improve signal in future experiments. Another method that has been employed in other RCA studies is the addition of a second primer not complimentary to the ligation site to increase amplification (24).

The method reported here is advantageous over thermal cycling methods in that it is isothermal; all elements are carried out at 30°C, and so only a simple heat block is required. Additionally, the Q-STAR probes are sequence-specific and since they are fluorogenic, a single tube is used for the entire experiment. The probes are versatile in multicolor readouts, offering the future possibility of detection of multiple miRNAs simultaneously using different fluorophores on separate Q-STAR probes (25). The use of chemical, rather than enzymatic, means for ligation and of Q-STAR probes should enhance the reproducibility and specificity of miRNA detection through RCA. Finally, this technique need not be confined to the detection of miRNA; it should be suitable for detection of other RNAs as well, as long as the site of ligation is positioned close to the 3’-end of the strand, so that replication can proceed using this as the primer.
SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table I and Supplementary Figures 1–7.

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