How to Perform miRacles: A Step-by-Step microRNA Detection Protocol Using DNA Nanoswitches

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MicroRNAs are short non-coding RNAs involved in post-transcriptional gene regulation, and are increasingly considered to be biomarkers for numerous biological processes and human diseases. Current techniques used for microRNA detection can be expensive and labor-intensive, and typically require amplification, labeling, or radioactive probes. In this protocol, we describe a DNA nanoswitch–based microRNA detection assay termed "miRacles": microRNA-activated conditional looping of engineered switches. This method uses conformationally responsive DNA nanoswitches that detect the presence of specific microRNAs with a simple and unambiguous gel-shift assay that can be performed on the benchtop. The assay is low cost, minimalistic, and capable of direct detection of specific microRNAs in unprocessed total RNA samples, with no enzymatic amplification, labeling, or special equipment. The protocol for detection of microRNAs in total RNA can be completed in as little as a few hours, making this assay a compelling alternative to qPCR and Northern blotting.

Basic Protocol 1: Preparation of DNA nanoswitches
Basic Protocol 2: Detection of microRNAs from total RNA samples
Support Protocol 1: Optional nanoswitch purification by PEG precipitation
Support Protocol 2: Optional nanoswitch purification by liquid chromatography

Keywords: biosensing • DNA nanoswitch • DNA nanotechnology • microRNA detection • miRacles • Northern blotting • qPCR • total RNA

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INTRODUCTION

MicroRNAs are short, noncoding RNAs (18 to 25 nt) that repress gene expression at the post-transcriptional level (Bartel, 2004). Since the first discovery of microRNAs in the early 1990s (Lee, Feinbaum, & Ambros, 1993), scientists have identified nearly 2000 microRNAs in the human genome, predicted to regulate more than 60% of genes (Friedman, Farh, Burge, & Bartel, 2009). MicroRNAs are known to play key roles in diverse biological processes including embryogenesis, organ development, proliferation, differentiation, apoptosis, metabolism, and homeostasis (Ebert & Sharp, 2012; Vidigal & Ventura,

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2015). Due to involvement in such biological processes, microRNAs are now studied as potential diagnostic biomarkers for various diseases. Expression levels of individual microRNAs in tissues, cells, and bodily fluids can be monitored to track cellular events or diagnose onset of diseases (Allegra et al., 2012; Cortez et al., 2011), highlighting the importance of a simple and sensitive method for microRNA detection and quantification.

MicroRNAs can be collected from biofluids including blood, urine, and saliva, among others, and maintain stability within microvesicles such as exosomes as well as microparticles and apoptotic bodies (Weber et al., 2010). Several recent studies have shown examples where microRNAs can be detected at earlier stages of diseases (Zhang et al., 2015). Differential expression of microRNAs can also be found in normal and diseased cells and tissues, serving as useful biomarkers for different cellular events and disease diagnosis, prognosis, and treatment monitoring. These criteria make microRNAs useful biomarkers, with many new studies identifying and validating unique microRNA signatures in biological processes and diseases.

MicroRNA detection is challenging due to low abundance, small size, and sequence similarities. MicroRNAs comprise about 0.01% of total RNA (Dong et al., 2013), and individual microRNA levels vary widely from a few copies to tens of thousands of copies per cell (Bartel, 2004). Furthermore, microRNAs within a family can differ by as little as a single nucleotide (Ryan, Robles, & Harris, 2010), and yet each specific microRNA can be differentially regulated during cellular processes or in disease conditions. Thus, microRNA detection strategies must be highly specific and thereby able to correctly identify a few target molecules among an abundance of similar RNA molecules.

Traditional methods for detection of microRNAs include Northern blotting, quantitative real-time PCR (qRT-PCR), next-generation sequencing, and microarray-based hybridization (Baker, 2010; Dong et al., 2013; Hunt, Broyles, Head, & Deo, 2015). Of these, only Northern blotting detects native microRNA directly, while the others rely on additional labeling or amplification steps. These approaches can present significant tradeoffs between cost, complexity, and performance. Additionally, some of these methods require specialized equipment and/or skilled personnel, and can involve complex and time-consuming procedures. We developed a relatively simple DNA-based device that can be used to detect microRNAs in total RNA samples from cells or tissues (Chandrasekaran, MacIsaac, et al., 2019). Our assay, termed *miRacles* (microRNA activated conditional looping of engineered switches), uses a "smart reagent" comprising rationally designed DNA nanoswitches to enable simple and low-cost detection of native microRNAs without specialized equipment (Fig. 1; Chandrasekaran, 2019).

The DNA nanoswitch is a linear duplex that forms a loop in the presence of a target microRNA (Fig. 2). The nanoswitch is constructed using DNA self-assembly techniques (Rothemund, 2006) and formed by hybridizing short oligonucleotides that are complementary to a single-stranded DNA scaffold (7249 nt). Two distant "detector" strands (separated by $\sim$2500 nt) are designed to contain overhangs complementary to different segments (typically halves) of the target microRNA. Recognition and binding of the microRNA reconfigures the switch from the linear "off" state to the looped "on" state. The two states can be quantified using standard agarose gel electrophoresis and gel stains, where the detection signal arises from the integrated intensity of the looped nanoswitch. The signal is provided by thousands of dye molecules intercalated into the nanoswitch, with the loop triggered by a single microRNA. This strategy provides an inherently strong signal originating from the nanoswitch rather than the target, and eliminates the need for amplification or labeling. Using this approach, we have demonstrated microRNA detection in the sub-attomole range (Chandrasekaran, MacIsaac, et al., 2019). To give perspective for potential users of the approach, this corresponds to a few copies per cell for a

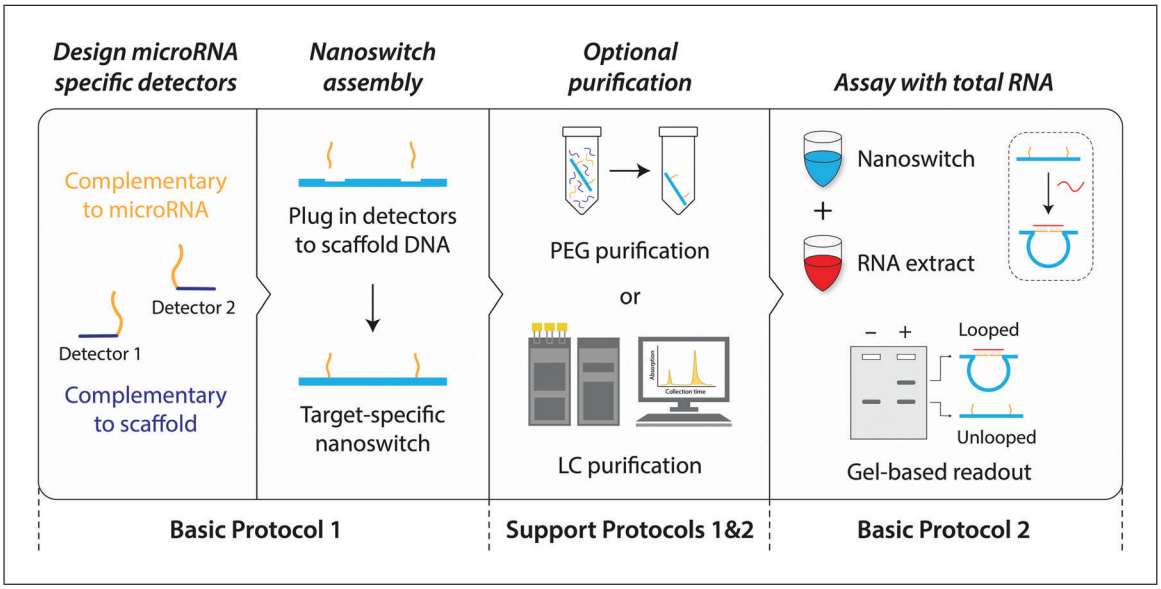

Figure 1 Workflow of the DNA nanoswitch–based *miRacles* assay for detecting microRNAs from total RNA samples.

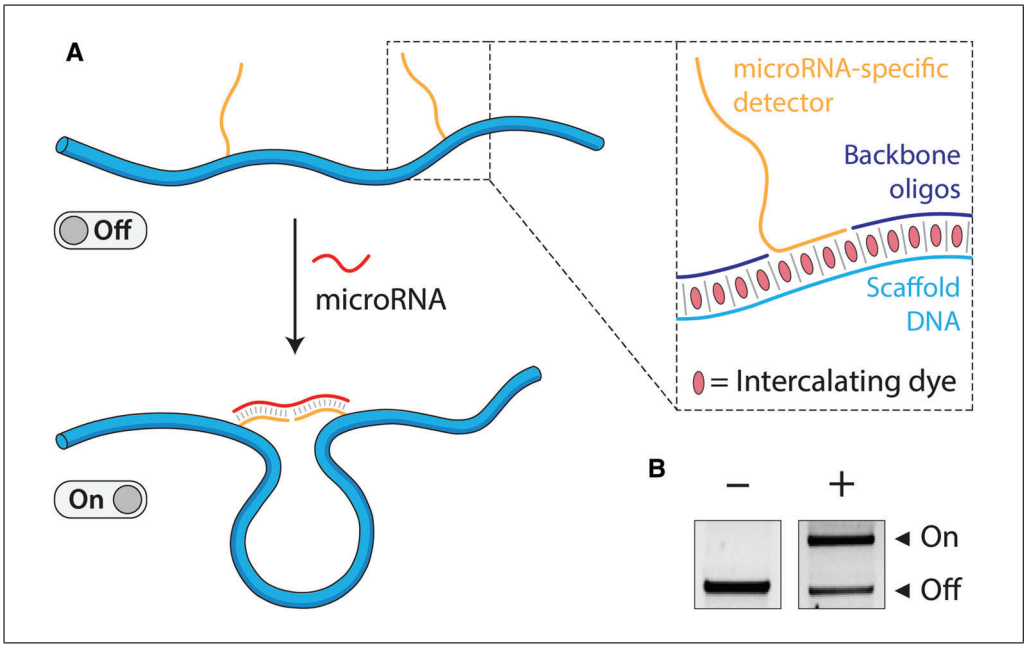

Figure 2 DNA nanoswitch design and operation. (**A**) The DNA nanoswitch is a long duplex with two single-stranded extensions that are complementary to the microRNA target. (**B**) Binding of target microRNA to the nanoswitch causes a conformational change that can be read out on an agarose gel. Figure reproduced from Chandrasekaran et al. (2019).

500 ng total RNA sample from a mammalian cell line. While not as sensitive as qPCR for microRNAs of extremely low abundance, it is orders of magnitude more sensitive than Northern blotting.

The protocols described here can be implemented in almost any biology or chemistry lab with a regular workflow such as pipetting mixtures and running agarose gels. The method requires an initial investment in the DNA strands required to make the nanoswitches. Our lab is committed to supporting the use of the miRacles assay, and will honor reasonable requests for materials in the form of "starter kits" containing the backbone oligonucleotides so that users can try the protocol with low up-front costs. Basic Protocol 1 describes the construction of DNA nanoswitches. The protocol can be used to create

nanoswitches specific to any target microRNA just by changing the detector strands. The prepared nanoswitches can be optionally purified by either PEG precipitation or using liquid chromatography as described in Support Protocol 1 or 2. Basic Protocol 2 provides a step-by-step instruction for performing the miRacles assay to detect a specific microRNA in total RNA samples from cells or tissues. Presence of the target microRNA is indicated by the appearance of the "on" band.

NOTE: In this article, we provide details about specific products and vendors we use, but similar materials may be obtained from other vendors. In some cases, we note where specific vendors are better.

BASIC PROTOCOL 1

PREPARATION OF DNA NANOSWITCHES

DNA nanoswitches are assembled from a scaffold DNA (M13 viral genome) and short complementary strands that make the nanoswitch a duplex. To make the nanoswitch, the first step is to linearize the circular M13 scaffold strand, followed by hybridization with backbone oligonucleotides and detector strands. Depending on the purpose of detection, the nanoswitches can be optionally purified by PEG precipitation (Support Protocol 1; Koussa, Sotomayor, & Wong, 2014) or by liquid chromatography (Support Protocol 2; Halvorsen, Kizer, Wang, Chandrasekaran, & Basanta-Sanchez, 2017) before use in detecting microRNAs.

Materials

250 μg/ml circular single-stranded M13 DNA (New England Biolabs, cat. no. N4040S); this corresponds to ∼100 nM

We have found that circular M13 bought commercially has some amount of linear DNA as a contaminant. This will affect nanoswitch efficiency since the linear cut site is random. We have found that M13 from NEB contains minimal linearized DNA compared to other vendors.

10× CutSmart buffer (New England Biolabs, cat. no. B7204S; included with *Bts*CI enzyme purchase)

100 μM synthetic DNA complementary to the *Bts*CI restriction site on the M13 ("cut site oligo" sequence in the recipe for "Detector strands and other oligonucleotides" in Reagents and Solutions"): can be ordered from Integrated DNA Technologies (IDT) or other oligo synthesis company—we typically order the smallest scale (25 nmol) with standard desalting

Nuclease-free water (ultrapure distilled water, DNase and RNase free, Invitrogen, cat. no. 10977-015)

*Bts*CI restriction enzyme (20,000 U/ml; New England Biolabs, cat. no. R0647S)

Oligonucleotide mixtures containing backbone strands and detector strands (see recipe for "Detector strands and other oligonucleotides" in Reagents and Solutions)

1× PBS working solution diluted in nuclease free water

PCR tubes

Thermal cycler (e.g.: BioRad T100 Thermal Cycler)

Designing microRNA-specific detectors

The nanoswitch can be considered as a "plug-and-play" device with customizable detectors for any microRNA. The detector sequences can be changed and the detectors incorporated into the nanoswitch using the following procedure.

1. Choose which microRNA you need to detect. The sequence of this microRNA should be known.
2. Split the sequence into two parts (typically halves, shown as *x* and *y* in Figure 3A). Get the complementary sequences to these halves.

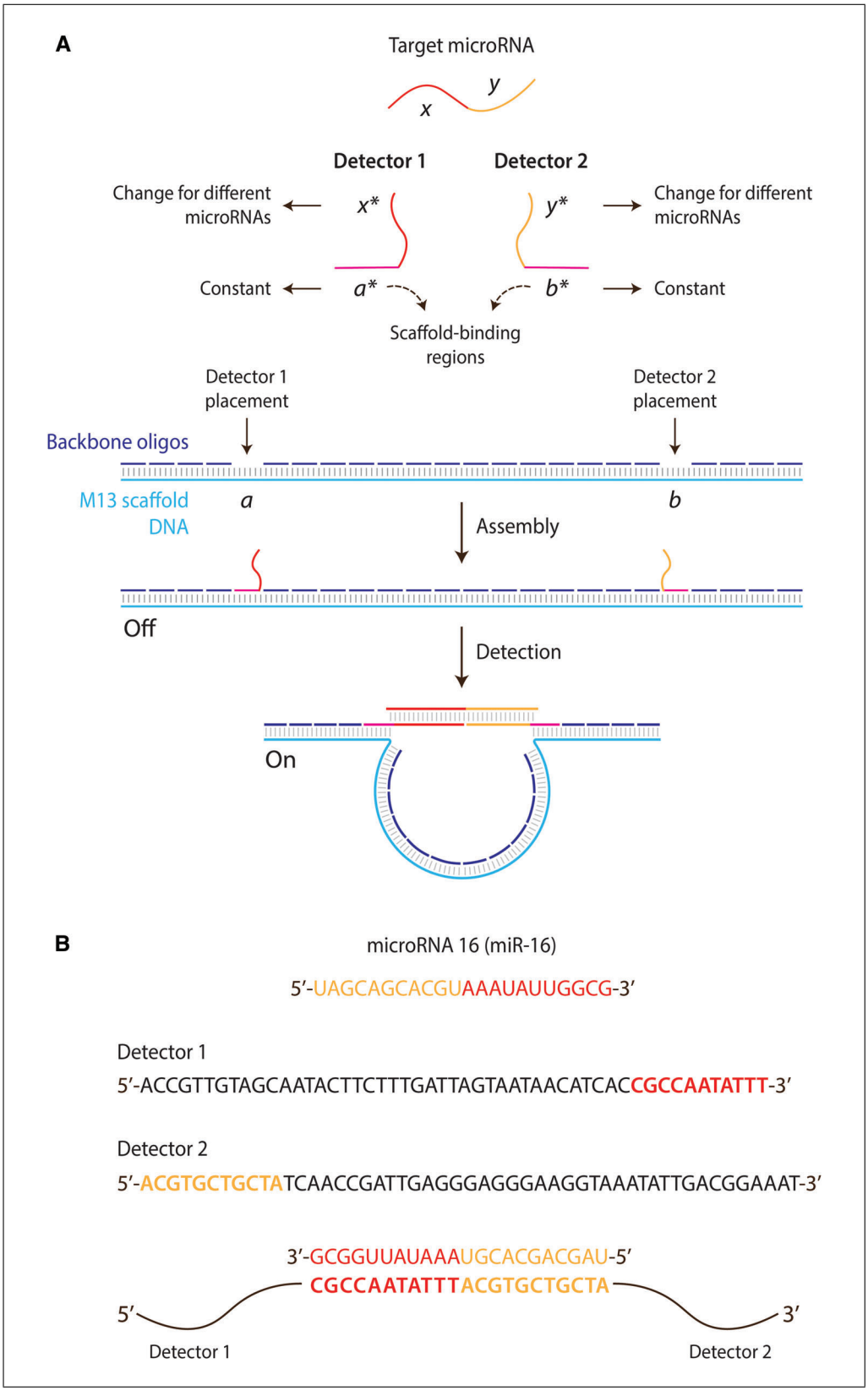

Figure 3 Designing microRNA-specific detectors. (**A**) Detectors 1 and 2 contain sequences that are complementary to parts of the target microRNA; complementary regions are shown in same color, red to red (*x*/*x**) and orange to orange (*y*/*y**). The other part of the detector binds to the scaffold to be incorporated into the nanoswitch. This sequence remains constant (*a*/*a** and *b*/*b**). To create detectors specific to a microRNA, only the regions *x** and *y** need to be changed. (**B**) An example sequence design shown with miR-16 as a model system. Complementary regions are denoted using the same color scheme as in (**A**).

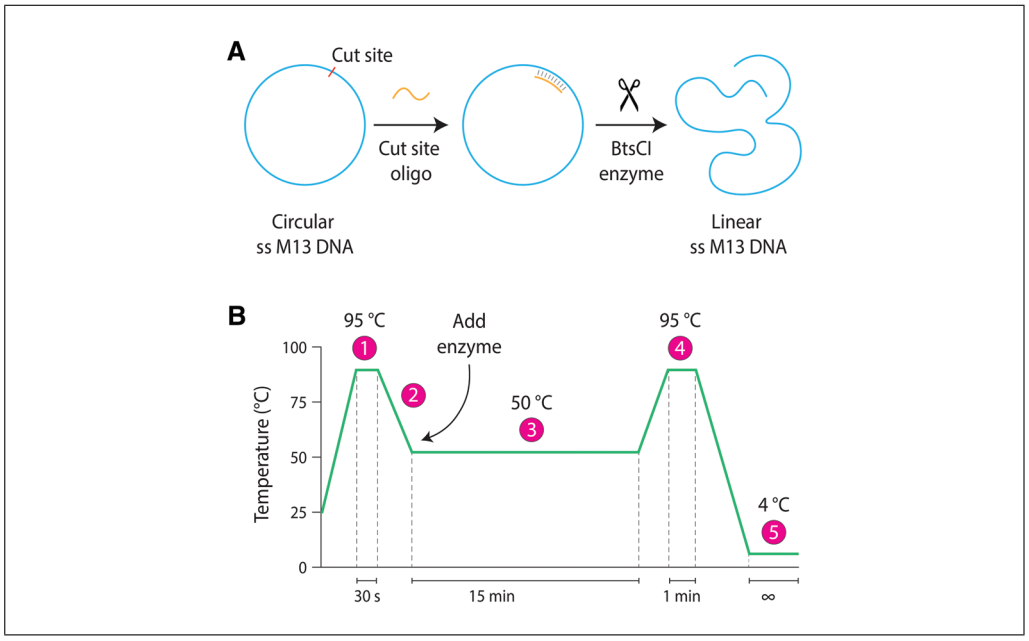

Figure 4 Linearization of circular M13 scaffold DNA. (**A**) Linearization of single-stranded circular M13 DNA. (**B**) Thermal cycler program for annealing of cut site oligo and *Bts*CI restriction enzyme activity.

3. Append the sequences to the scaffold-binding region of the detector in the correct 5′ to 3′ polarity (example shown in Fig. 3B).
4. Order detectors 1 and 2 with these sequences from companies such as Integrated DNA Technologies (IDT).
5. Use detector sequences to make nanoswitches according to the self-assembly protocol below.

Linearization of M13 scaffold strand (time: 20 min)

The first step is to linearize single-stranded circular M13 DNA using a restriction enzyme. A short DNA strand complementary to the restriction site for *Bts*CI enzyme is hybridized with the circular M13 DNA. The enzyme is then added to convert it to a linear strand (Fig. 4A).

6. Hybridize cut site oligo to M13 scaffold by mixing the following in a PCR tube:

   5 µl circular single-stranded M13 DNA (250 µg/ml)
   2.5 µl 10× CutSmart buffer
   1 µl cut site oligo (100 µM)
   16.5 µl nuclease-free water.

   We have scaled this up as much as four times, resulting in 100 µl of final product, with no obvious reduction in linearization efficiency.

7. Pipette to mix thoroughly, being sure to avoid air bubbles.
8. Program thermal cycler and incubate the mixture with the following program:

   95°C for 30 s
   50°C for 15 min (pause, see step 9)
   95°C for 1 min
   4°C (hold).

9. When the thermal cycler reaches 50°C, pause the program and add 1 µl of the *Bts*CI enzyme to the mixture (Fig. 4B).

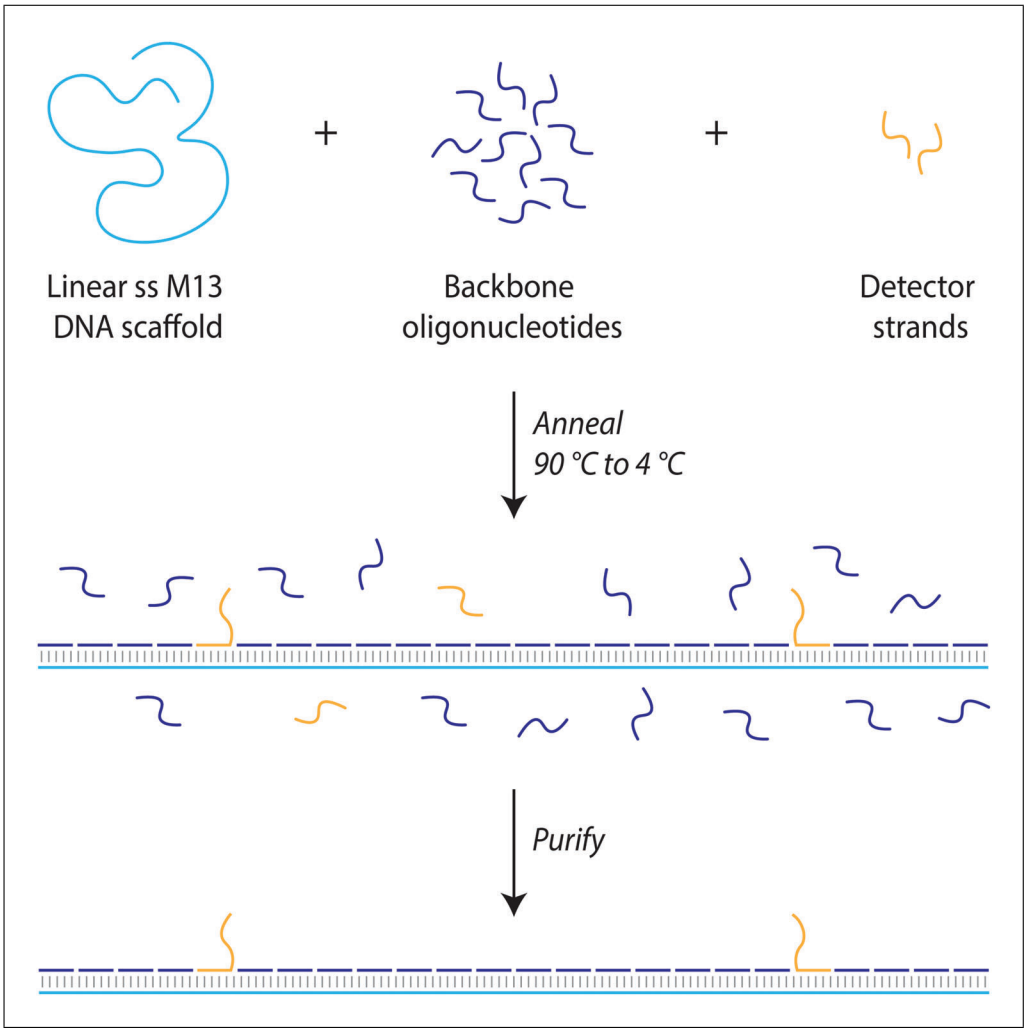

Figure 5 Nanoswitch preparation and purification.

CRITICAL STEP: *Mix thoroughly with a pipette to ensure that enzyme is well mixed. The enzyme is in glycerol and will sink to the bottom if not mixed well. It is best to use a pipette set to ~10 µl to do this, as a 1-µl pipette may not provide adequate mixing. Failure to do this may greatly lower linearization efficiency.*

10. Resume the thermal cycler program.

    For convenience, you may want to set the thermal cycler to hold at 4°C after completion.

11. This will result in 25 µl of ~20 nM linearized M13. The linearized M13 can be stored at 4°C for multiple uses in the future.

Self-assembly of DNA nanoswitch (time: ~90 min)

Now that the linear single-stranded scaffold has been made, you will need the set of oligonucleotides that are complementary to the scaffold. We call these "backbone" oligonucleotides, and they can be bought pre-mixed, or purchased individually and then mixed, for assembling the nanoswitch. Two regions on the scaffold are left single-stranded to bind the detector strands. Part of these detector strands bind to the M13 scaffold at pre-defined locations, and the other segment is complementary to parts of the target microRNA (Fig. 2). This step involves the construction of the DNA nanoswitch. The components include the linear “scaffold” DNA, a set of complementary “backbone” oligonucleotides, and a pair of “detector” strands specific to the target microRNA to be detected (Fig. 5).

12. Make a mixture of all the backbone oligonucleotides (called "backbone mix"; see recipe for "Detector strands and other oligonucleotides" in Reagents and Solutions for sequences). If the 121 backbone oligonucleotides were bought as a mixture, hydrate with nuclease-free water to achieve a total oligonucleotide concentration of 100 μM. If they were bought in separate tubes, hydrate each strand with nuclease-free water to 100 μM and mix strands in equal volumes (e.g., 2 μl from each oligo).

    This mix is common to preparing any nanoswitch and can be used to build a nanoswitch to detect any microRNA. Only the detector strands need to the changed (see step 15).

13. Hydrate the detector strands with nuclease-free water to 100 μM.

14. Mix the following to make the "detector mix" (see recipe for "Detector strands and other oligonucleotides" in Reagents and Solutions for sequences). To design detectors for any specific microRNA, follow Figure 3.

    5 μl of detector 1
    5 μl of detector 2.

15. Mix the following to make the "total oligo mix." This mix will contain 123 oligos.

    27.3 μl of backbone mix
    0.5 μl of detector mix.

    These volumes can be scaled up or down, but we recommend not pipetting less than 0.5 μl of the detector mix, to ensure accuracy. Oligo mixes can be stored frozen for years.

16. Mix the "total oligo mix" and linearized M13 scaffold strand in a PCR tube in the following amounts:

    5 μl of linearized single-stranded M13 DNA (from step 11)
    1.2 μl of total oligo mix (from step 15).

    This is essentially like adding 0.01 μl of each oligonucleotide at 100 μM, which gives a 10-fold excess to the 5 μl of 20 nM scaffold. These volumes can be scaled up as needed, but contain enough nanoswitch for about 100 reactions. We have tested a 4× scale-up without problems.

17. Hybridize the scaffold and oligo mix in a thermal cycler using the following protocol:

    Step 1: Bring to 90°C and hold for 30 s
    Step 2: Cool to 4°C at the rate of 1°C per min.

    For convenience you may want to set the thermocycler to hold at 4°C after completion. Once made, the nanoswitches can be stored at 4°C until purification or further use.

18. To use unpurified nanoswitches for experiments, dilute nanoswitches 40× in 1× PBS.

    The nanoswitches can optionally be purified depending on the purpose of use (for example, experiments requiring high sensitivity). If you want to purify nanoswitches, follow Support Protocol 1 for PEG precipitation or Support Protocol 2 for liquid chromatography.

BASIC PROTOCOL 2

DETECTION OF microRNA FROM TOTAL RNA SAMPLES

The prepared nanoswitches (unpurified or purified) can now be used to detect specific microRNAs from cellular RNA extracts. You can use either small RNA or total RNA extracted from cells or tissues. The following protocol describes detection of a specific microRNA sequence from a total RNA sample.

Additional Materials *(also see Basic Protocol 1)*

Total RNA (your sample): we used total RNA from MCF7 cell line as a model system here
10× PBS (phosphate-buffered saline), pH 7.4 (VWR, cat. no. 75800-994)
1× PBS working solution diluted in nuclease free water
GelRed nucleic acid stain (1:300 dilution; see recipe)
Nanoswitch prepared using Basic Protocol 1 (∼400 pM)
Agarose (molecular biology grade, Sigma, cat no. A9539)
0.5× TBE running buffer (see recipe)
6× loading dye solution (see recipe)
Synthetic DNA or RNA sequence for positive spike (25 nM; see recipe for "Detectors and other oligonucleotides" in Reagents and Solutions for sequence)

50-ml Erlenmeyer flask
Magnetic stir bar
Aluminum foil
Hot plate with stirrer
Mittens
Gel electrophoresis chamber and comb (Thermo Scientific Owl EasyCast B1A Mini Gel)
Power supply for gel electrophoresis
GelDoc imaging station or similar (Bio-Rad Gel Doc XR+ imager)

NOTE: We have tested agarose from different vendors and found this product to have the least background when imaging the gel. For experiments requiring sensitive detection, we recommend this particular product/vendor.

Incubate total RNA with nanoswitch (time: 2 to 12 hr)

1. Dilute total RNA samples to 500 ng/μl with 1× PBS.

   For a typical assay, use 250 to 500 ng of total RNA per lane.

2. Incubate total RNA with GelRed (1:300 dilution in 1× PBS) for 1 hr: add 1 μl of GelRed diluted 1:300 to 1 μl of total RNA sample (at 500 ng/μl).

   We have optimized this 3.3× GelRed concentration for better detection. Depending on the sample, the amount of GelRed can be varied (1× to 10×).

3. Prepare synthetic DNA or RNA controls for the specific microRNA you are detecting. Dilute the strand to 25 nM concentration in nuclease-free water.

4. Make control and test lanes by following the instructions in Table 1 for each lane.

5. Once mixed, incubate the samples in a thermal cycler in an overnight annealing ramp (∼12.5 hr total).

   Step 1: Bring to 40°C and hold for 30 s
   Step 2: Cool to 25°C with 5 min for each 0.1°C.

   We have also tested a 2-hr annealing protocol from 40°C to 25°C for successful microRNA detection from total RNA. If the target microRNA is known to exist in higher abundance, the shorter annealing ramp can be used.

Prepare agarose gel (time: 45 min)

6. Mix the following to make a 0.8% agarose gel:

   0.2 g of ultrapure agarose in a 50-ml Erlenmeyer flask
   25 ml of 0.5× TBE.

Table 1 Preparation of Total RNA and Control Samples for microRNA Detection

	Buffer controls		In total RNA		
Components	Negative control	Positive control	Positive spike	Total RNA + nanoswitch	Only total RNA
Nanoswitch	2 μl	2 μl	2 μl	2 μl	—
DNA or RNA control	—	1 μl	1 μl	—	—
Total RNA	—	—	1 μl	1 μl	1 μl
GelRed (300× dilute)	1 μl	1 μl	1 μl	1 μl	1 μl
1× PBS	7 μl	6 μl	5 μl	6 μl	8 μl
Total	10 μl	10 μl	10 μl	10 μl	10 μl

7. Add a magnetic stir bar to the flask and cover the top of the flask with aluminum foil to prevent vapor from escaping.
8. Place the Erlenmeyer flask on a hot plate with stirring capability.
9. Bring to a rolling boil while stirring, and allow to boil for 30 s.
10. Let the flask cool until it can be held comfortably in a gloved hand for a few seconds. This can be accelerated by running the flask under cold water if needed.

    If the gel cools too much, it will not pour evenly. It is better to err on the side of too hot than too cold—but much too hot will warp the plastic of the gel box.

11. Pour the gel into a molding tray with a gel comb and allow to solidify (30 to 45 min).

Run nanoswitch samples on agarose gel (time: 50 min)

The prepared samples will be run on an agarose gel to read out detection of the target microRNA.

12. Add 2 μl of 6× loading dye to each of the samples prepared in step 5.
13. Fill the gel box with the 0.5× TBE running buffer (to the fill line).
14. Load 10 μl of each sample into the wells in the gel.
15. Run the gel by applying 75 V for 45 min.

    We use the Owl Easy Cast B1A gel box (ThermoFisher Scientific) and optimized gel-running conditions, typically 75 V for 30 to 60 min. The voltage and running time might have to be optimized based on the type of agarose gel electrophoresis equipment available in the laboratory.

Image the gel

16. Once the gel has been run, remove from the gel box and place on the imaging plate on a Bio-Rad Gel Doc XR+ gel imager (or any gel imager available). Image using the gel-analysis tool in the Image Lab software package available with BioRad Gel Doc XR+ (or the software available with your gel imager).

Gel image analysis and quantification

The expected results from the total RNA experiment are shown in Figure 6. Gel images can be quantified using the Image Lab software that is provided with the BioRad Gel Doc XR+ gel imager (*http://www.bio-rad.com/en-us/product/image-lab-software*). Another gel image–analysis software application is ImageJ (*https://imagej.nih.gov/ij/*).

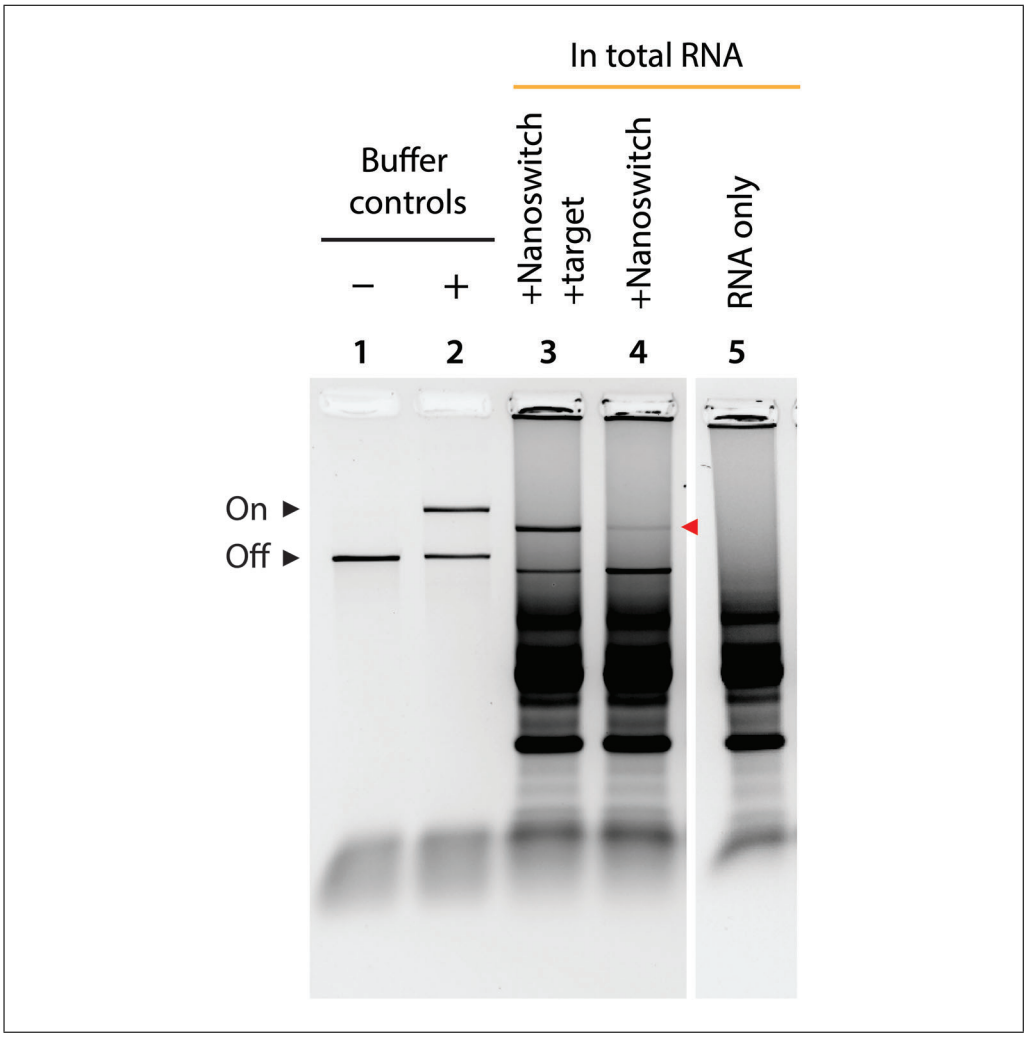

Figure 6 Detection of microRNA 16 (miR-16) from 500 ng of MCF7 cell line total RNA. Figure adapted from Chandrasekaran et al. (2019).

Both software packages are freely available to download. We quantified the intensity of the looped band to analyze changes in microRNA levels in different samples.

OPTIONAL NANOSWITCH PURIFICATION BY PEG PRECIPITATION

SUPPORT PROTOCOL 1

The backbone and detector strands are added in excess during construction of the nanoswitches and can be optionally purified out (Fig. 7A). This is done by precipitation of the larger nanoswitches from remaining excess strands using 4% polyethylene glycol (PEG, see Reagents and Solutions). Large pieces of DNA such as the nanoswitch will precipitate out, while smaller pieces of DNA such as the oligonucleotides will remain in the supernatant (Fig. 7B). The purification process can alternatively be done using liquid chromatography following Support Protocol 2, depending on instrument availability.

The time for completing this protocol is ~70 min.

Materials

Nanoswitch prepared using Basic Protocol 1, step 17
4% PEG 8000 solution in 30 mM $MgCl_2$ (freshly prepared, see recipe).
10× PBS (phosphate-buffered saline), pH 7.4 (VWR, cat. no. 75800-994)
1× PBS working solution diluted in nuclease-free water.

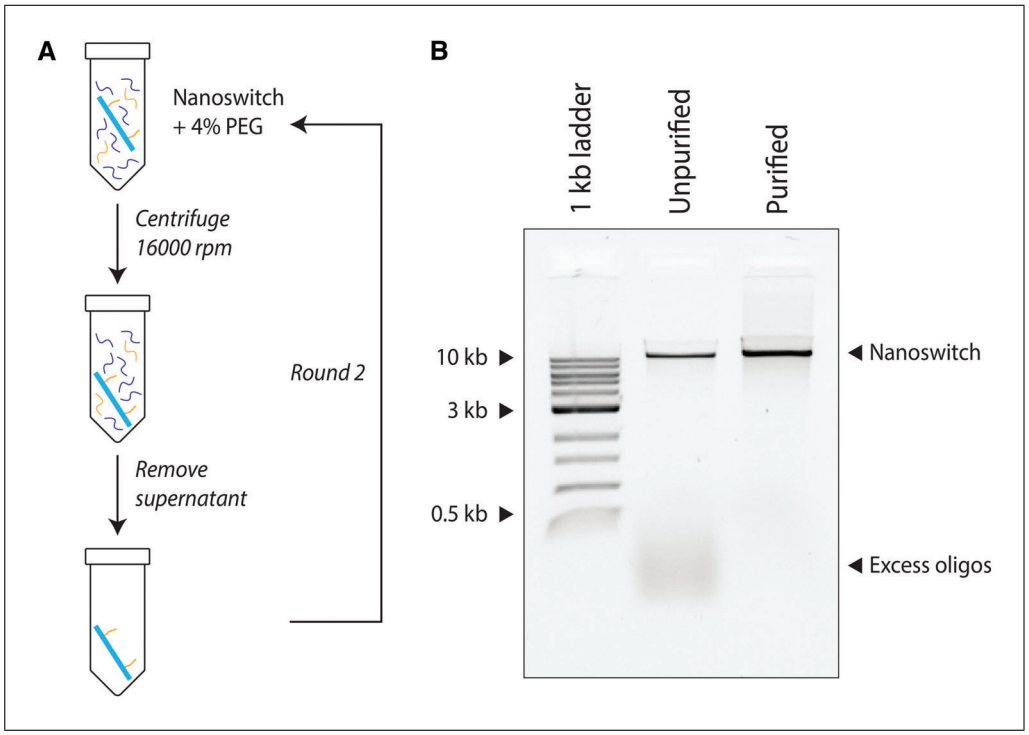

Figure 7 Purification of DNA nanoswitches by PEG precipitation. (**A**) Procedure to precipitate and remove excess oligos from DNA nanoswitches using PEG. (**B**) Agarose gel showing the unpurified and PEG-purified nanoswitches. Excess oligos are removed after purification, resulting in a slightly concentrated nanoswitch solution.

DNA LoBind tubes (Eppendorf, cat. no. 022431021)
Microcentrifuge (e.g.: Beckman Coulter Microcentrifuge 16)

1. Transfer the assembled nanoswitch (~6 µl) to a new 1.5-ml DNA LoBind tube.

   Using DNA LoBind tubes will prevent DNA from sticking to the tubes, and provides higher purification yields.

2. Add 194 µl of 4% PEG and mix gently but thoroughly (by pipetting, do not vortex).
3. Centrifuge for 30 min at 14,800 rpm (or similar maximum setting) at room temperature.
4. Carefully remove the top 180 µl using a 200-µl pipette.

   CRITICAL STEP: *This should be done very carefully, as it is easy to disturb the pellet. It should take roughly 30 s to slowly pipette out the supernatant in one smooth draw. It helps to always pipette near the fluid/air boundary so as to be as far away from the pellet as possible (you will not see the DNA pellet).*

5. Add 180 µl of 4% PEG and mix gently but thoroughly.
6. Centrifuge for 30 min at 14,800 rpm at room temperature.
7. Carefully remove the top 180 µl using a 200-µl pipette.

   The remaining 20 µl should contain the DNA nanoswitch free of any detectable amount of free-floating oligonucleotides (Fig. 7B).

8. Dilute nanoswitches to working concentration (200 to 500 pM) by adding 40 to 60 µl of 1× PBS.

   The concentration of diluted nanoswitches can be tested on an agarose gel for sharp nanoswitch bands, and the concentration adjusted by adding more 1× PBS.

OPTIONAL NANOSWITCH PURIFICATION BY LIQUID CHROMATOGRAPHY

Purification by liquid chromatography (LC) is fast and efficient, but we recognize that it is dependent on the instruments available in each lab. We use a Waters H-class UPLC, but our protocol can be adapted to other instruments that can inject and collect small volumes. We have made some optimizations to our published protocol (Halvorsen et al., 2017), and briefly mention the procedure here (Fig. 8). This protocol can be adapted to many LC instruments and purification columns, and optimized as required.

The LC protocol uses an isocratic method to enable size-based separation of nanoswitches from excess oligos as they flow through the column. The column we use is an Agilent PLRP-S column with 8-µm particles, 1000 Å pore size, and 2.1 × 50 mm dimensions. The nanoswitch mixture is flowed through the column with a 90:10 mixture of 10 mM ammonium acetate:HPLC grade acetonitrile, and nanoswitches are eluted prior to oligos as in size-exclusion chromatography. Alternatively, nanoswitches can be purified manually by the PEG precipitation procedure described in Support Protocol 1.

The time for completing this protocol is ∼15 min.

Materials

10 mM ammonium acetate (Millipore Sigma, cat. no. 631-61-8)
HPLC-grade acetonitrile (100% and 50%; Fisher Scientific, cat. no. A998-1)
Nanoswitch prepared using Basic Protocol 1

0.2-µm sterile filters
HPLC or UPLC instrument (e.g., Waters H-class UPLC)

1. Prepare LC buffer as a 90:10 mixture of 10 mM ammonium acetate and HPLC-grade acetonitrile. We recommend filtering the solution through a 0.2-µm filter prior to use, and changing the buffer weekly.
2. Prepare a sample dilution buffer as a 70:30 mixture of 10 mM ammonium acetate and HPLC-grade acetonitrile. Mix equal parts of nanoswitch and the 70:30 ammonium acetate:acetonitrile solution to prepare sample for injection.

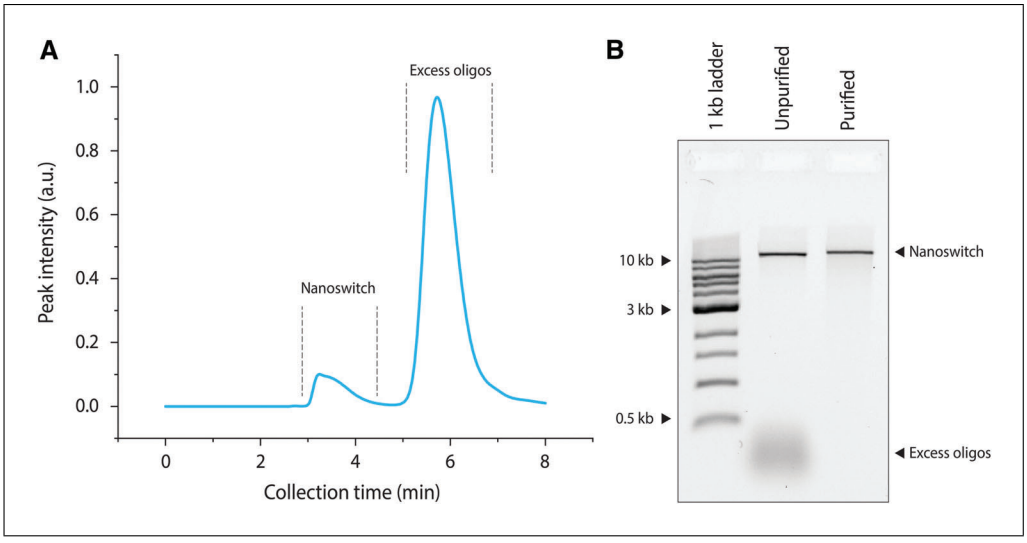

Figure 8 Purification of DNA nanoswitches by liquid chromatography. (**A**) Chromatogram showing the peaks for the nanoswitch and excess oligos eluting at different times. (**B**) Agarose gel showing the unpurified and LC-purified nanoswitches. Excess oligos are removed after purification.

This step is important to ensure that the injected sample has similar (or higher) acetonitrile than the flow condition, to ensure no sticking to the column.

3. Start HPLC with 0.02 ml/min flow of LC buffer and allow column to equilibrate for several minutes (column temperature of 25°C). The pressure should be steady to within a few psi (expect pressures in the 60 to 80 psi range depending on exact system conditions).

   Higher pressures can indicate column degradation or clogging. Increasing pressure over time can be avoided by maintaining clean samples and not overloading the column. We have performed over 1000 injections on a single column when it was carefully maintained.

4. Inject 1 to 10 µl of the nanoswitch mixture into the column.

5. Collect the first peak, which contains only the nanoswitch. The excess oligos will elute as a second peak. Exact timing may vary depending on the HPLC/UPLC system used and tubing volumes, etc.

6. Before subsequent runs, flush column with 50% acetonitrile for a few minutes to fully purge the column.

REAGENTS AND SOLUTIONS

Acetonitrile, 50%

500 ml acetonitrile (HPLC-grade)
500 ml deionized water
Store up to 6 months at room temperature

Ammonium acetate, 1 M

77.08 g ammonium acetate
Bring volume to 1 L
Store up to 6 months at 4°C

Ammonium acetate, 10 mM

10 ml 1 M ammonium acetate (see recipe)
990 ml deionized water
Store up to 6 months at 4°C

Detector strands and other oligonucleotides

see Tables 2 and 3.

Table 2 List of Detectors and Target microRNA Sequences (Written From 5′ to 3′)

MicroRNA, detector, and cut site oligos		
#	Sequence	Length
Detector 1	**ACCGTTGTAGCAATACTTCTTTGATTAGTAATAACATCAC**CGCC AATATTT	51
Detector 2	ACGTGCTGCTA**TCAACCGATTGAGGGAGGGAAGGTAAATATTGA CGGAAAT**	51
Target microRNA (miR-16)	UAGCAGCACGUAAAUAUUGGCG	22
*Bts*CI cut site oligo	CTACTAATAGTAGTAGCATTAACATCCAATAAATCATACA	40

[a]Regions in bold indicate the region of the detectors that bind to the scaffold. Red and blue regions are single-stranded extensions on detectors that are complementary to two halves of the target microRNA.

Table 3 List of Backbone Oligonucleotide Sequences (Written From 5′ to 3′)

Backbone oligonucleotides		
#	Sequence	Length
1	AGAGCATAAAGCTAAATCGGTTGTACCAAAAACATTATGACCCTGTA ATACTTTTGCGGG	60
2	AGAAGCCTTTATTTCAACGCAAGGATAAAAATTTTTAGAACCCTCAT ATATTTTAAATGC	60
3	AATGCCTGAGTAATGTGTAGGTAAAGATTCAAAAGGGTGAGAAAGGC CGGAGACAGTCAA	60
4	ATCACCATCAATATGATATTCAACCGTTCTAGCTGATAAATTAATGC CGGAGAGGGTAGC	60
5	TATTTTTGAGAGATCTACAAAGGCTATCAGGTCATTGCCTGAGAGTC TGGAGCAAACAAG	60
6	AGAATCGATGAACGGTAATCGTAAAACTAGCATGTCAATCATATGTA CCCCGGTTGATAA	60
7	TCAGAAAAGCCCCAAAAACAGGAAGATTGTATAAGCAAATATTTAAA TTGTAAACGTTAA	60
8	TATTTTGTTAAAATTCGCATTAAATTTTTGTTAAATCAGCTCATTTT TTAACCAATAGGA	60
9	ACGCCATCAAAAATAATTCGCGTCTGGCCTTCCTGTAGCCAGCTTTC ATCAACATTAAAT	60
10	GGATAGGTCACGTTGGTGTAGATGGGCGCATCGTAACCGTGCATCTG CCAGTTTGAGGGG	60
11	ACGACGACAGTATCGGCCTCAGGAAGATCGCACTCCAGCCAGCTTTC CGGCACCGCTTCT	60
12	GGTGCCGGAAACCAGGCAAAGCGCCATTCGCCATTCAGGCTGCGCAA CTGTTGGGAAGGG	60
13	CGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGG ATGTGCTGCAAGG	60
14	CGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAA AACGACGGCCAGT	60
15	GCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTAC CGAGCTCGAATTC	60
16	GTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCA CAATTCCACACAA	60
17	CATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAG TGAGCTAACTCAC	60
18	ATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGT CGTGCCAGCTGCA	60
19	TTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGC GCCAGGGTGGTTT	60
20	GTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATCC TGTTTGATGGTGG	60
21	TTCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGCCCGAGA TAGGGTTGAGTGT	60
22	TGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCA ACGTCAAAGGGCG	60

(*Continued*)

Table 3 List of Backbone Oligonucleotide Sequences (Written From 5′ to 3′), *continued*

Backbone oligonucleotides		
#	Sequence	Length
23	AAAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCA AATCAAGTTTTTT	60
24	GGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCC CCCGATTTAGAGC	60
25	TTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAG CGAAAGGAGCGGG	60
26	CGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCA CACCCGCCGCGCT	60
27	TAATGCGCCGCTACAGGGCGCGTACTATGGTTGCTTTGACGAGCACG TATAACGTGCTTT	60
28	CCTCGTTAGAATCAGAGCGGGAGCTAAACAGGAGGCCGATTAAAGGG ATTTTAGACAGGA	60
29	ACGGTACGCCAGAATCCTGAGAAGTGTTTTTATAATCAGTGAGGCCA CCGAGTAAAAGAG	60
30	TTGCCTGAGTAGAAGAACTCAAACTATCGGCCTTGCTGGTAATATCC AGAACAATATTAC	60
31	CGCCAGCCATTGCAACAGGAAAAACGCTCATGGAAATACCTACATTT TGACGCTCAATCG	60
32	TCTGAAATGGATTATTTACATTGGCAGATTCACCAGTCACACGACCA GTAATAAAAGGGA	60
33	CATTCTGGCCAACAGAGATAGAACCCTTCTGACCTGAAAGCGTAAGA ATACGTGGCACAG	60
34	ACAATATTTTTGAATGGCTATTAGTCTTTAATGCGCGAACTGATAGC CCTAAAACATCGC	60
35	CATTAAAAATACCGAACGAACCACCAGCAGAAGATAAAACAGAGGTG AGGCGGTCAGTAT	60
36	TAACACCGCCTGCAACAGTGCCACGCTGAGAGCCAGCAGCAAATGAA AAATCTAAAGCAT	60
37	CACCTTGCTGAACCTCAAATATCAAACCCTCAATCAATATCTGGTCA GTTGGCAAATCAA	60
38	CAGTTGAAAGGAATTGAGGAAGGTTATCTAAAATATCTTTAGGAGCA CTAACAACTAATA	60
39	GATTAGAGCCGTCAATAGATAATACATTTGAGGATTTAGAAGTATTA GACTTTACAAACA	60
40	CATTATCATTTTGCGGAACAAAGAAACCACCAGAAGGAGCGGAATTA TCATCATATTCCT	60
41	GATTATCAGATGATGGCAATTCATCAATATAATCCTGATTGTTTGGA TTATACTTCTGAA	60
42	TAATGGAAGGGTTAGAACCTACCATATCAAAATTATTTGCACGTAAA ACAGAAATAAAGA	60
43	AATTGCGTAGATTTTCAGGTTTAACGTCAGATGAATATACAGTAACA GTACCTTTTACAT	60
44	CGGGAGAAACAATAACGGATTCGCCTGATTGCTTTGAATACCAAGTT ACAAAATCGCGCA	60

(*Continued*)

Table 3 List of Backbone Oligonucleotide Sequences (Written From 5′ to 3′), *continued*

Backbone oligonucleotides		
#	Sequence	Length
45	GAGGCGAATTATTCATTTCAATTACCTGAGCAAAAGAAGATGATGAA ACAAACATCAAGA	60
46	AAACAAAATTAATTACATTTAACAATTTCATTTGAATTACCTTTTTT AATGGAAACAGTA	60
47	CATAAATCAATATATGTGAGTGAATAACCTTGCTTCTGTAAATCGTC GCTATTAATTAAT	60
48	TTTCCCTTAGAATCCTTGAAAACATAGCGATAGCTTAGATTAAGACG CTGAGAAGAGTCA	60
49	ATAGTGAATTTATCAAAATCATAGGTCTGAGAGACTACCTTTTTAAC CTCCGGCTTAGGT	60
50	GAAAACTTTTTCAAATATATTTTAGTTAATTTCATCTTCTGACCTAA ATTTAATGGTTTG	60
51	AAATACCGACCGTGTGATAAATAAGGCGTTAAATAAGAATAAACACC GGAATCATAATTA	60
52	CTAGAAAAAGCCTGTTTAGTATCATATGCGTTATACAAATTCTTACC AGTATAAAGCCAA	60
53	CGCTCAACAGTAGGGCTTAATTGAGAATCGCCATATTTAACAACGCC AACATGTAATTTA	60
54	GGCAGAGGCATTTTCGAGCCAGTAATAAGAGAATATAAAGTACCGAC AAAAGGTAAAGTA	60
55	ATTCTGTCCAGACGACGACAATAAACAACATGTTCAGCTAATGCAGA ACGCGCCTGTTTA	60
56	TCAACAATAGATAAGTCCTGAACAAGAAAAATAATATCCCATCCTAA TTTACGAGCATGT	60
57	AGAAACCAATCAATAATCGGCTGTCTTTCCTTATCATTCCAAGAACG GGTATTAAACCAA	60
58	GTACCGCACTCATCGAGAACAAGCAAGCCGTTTTTATTTTCATCGTA GGAATCATTACCG	60
59	CGCCCAATAGCAAGCAAATCAGATATAGAAGGCTTATCCGGTATTCT AAGAACGCGAGGC	60
60	ATTTTGCACCCAGCTACAATTTTATCCTGAATCTTACCAACGCTAAC GAGCGTCTTTCCA	60
61	GAGCCTAATTTGCCAGTTACAAAATAAACAGCCATATTATTTATCCC AATCCAAATAAGA	60
62	AACGATTTTTTGTTTAACGTCAAAAATGAAAATAGCAGCCTTTACAG AGAGAATAACATA	60
63	AAAACAGGGAAGCGCATTAGACGGGAGAATTAACTGAACACCCTGAA CAAAGTCAGAGGG	60
64	TAATTGAGCGCTAATATCAGAGAGATAACCCACAAGAATTGAGTTAA GCCCAATAATAAG	60
65	AGCAAGAAACAATGAAATAGCAATAGCTATCTTACCGAAGCCCTTTT TAAGAAAAGTAAG	60
66	CAGATAGCCGAACAAAGTTACCAGAAGGAAACCGAGGAAACGCAATA ATAACGGAATACC	60

(*Continued*)

Table 3 List of Backbone Oligonucleotide Sequences (Written From 5′ to 3′), *continued*

Backbone oligonucleotides		
#	Sequence	Length
67	CAAAAGAACTGGCATGATTAAGACTCCTTATTACGCAGTATGTTAGC AAACGTAGAAAAT	60
68	ACATACATAAAGGTGGCAACATATAAAAGAAACGCAAAGACACCACG GAATAAGTTTATT	60
69	TTGTCACAATCAATAGAAAATTCATATGGTTTACCAGCGCCAAAGAC AAAAGGGCGACAT	60
70	TCACCGTCACCGACTTGAGCCATTTGGGAATTAGAGCCAGCAAAATC ACCAGTAGCACCA	60
71	TTACCATTAGCAAGGCCGGAAACGTCACCAATGAAACCATCGATAGC AGCACCGTAATCA	60
72	GTAGCGACAGAATCAAGTTTGCCTTTAGCGTCAGACTGTAGCGCGTT TTCATCGGCATTT	60
73	TCGGTCATAGCCCCCTTATTAGCGTTTGCCATCTTTTCATAATCAAA ATCACCGGAACCA	60
74	GAGCCACCACCGGAACCGCCTCCCTCAGAGCCGCCACCCTCAGAACC GCCACCCTCAGAG	60
75	CCACCACCCTCAGAGCCGCCACCAGAACCACCACCAGAGCCGCCGCC AGCATTGACAGGA	60
76	GGTTGAGGCAGGTCAGACGATTGGCCTTGATATTCACAAACAAATAA ATCCTCATTAAAG	60
77	CCAGAATGGAAAGCGCAGTCTCTGAATTTACCGTTCCAGTAAGCGTC ATACATGGCTTTT	60
78	GATGATACAGGAGTGTACTGGTAATAAGTTTTAACGGGGTCAGTGCC TTGAGTAACAGTG	60
79	CCCGTATAAACAGTTAATGCCCCCTGCCTATTTCGGAACCTATTATT CTGAAACATGAAA	60
80	CCAGGCGGATAAGTGCCGTCGAGAGGGTTGATATAAGTATAGCCCGG AATAGGTGTATCA	60
81	CCGTACTCAGGAGGTTTAGTACCGCCACCCTCAGAACCGCCACCCTC AGAACCGCCACCC	60
82	TCAGAGCCACCACCCTCATTTTCAGGGATAGCAAGCCCAATAGGAAC CCATGTACCGTAA	60
83	CACTGAGTTTCGTCACCAGTACAAACTACAACGCCTGTAGCATTCCA CAGACAGCCCTCA	60
84	TAGTTAGCGTAACGATCTAAAGTTTTGTCGTCTTTCCAGACGTTAGT AAATGAATTTTCT	60
85	GTATGGGATTTTGCTAAACAACTTTCAACAGTTTCAGCGGAGTGAGA ATAGAAAGGAACA	60
86	ACTAAAGGAATTGCGAATAATAATTTTTTCACGTTGAAAATCTCCAA AAAAAAGGCTCCA	60
87	AAAGGAGCCTTTAATTGTATCGGTTTATCAGCTTGCTTTCGAGGTGA ATTTCTTAAACAG	60
88	CTTGATACCGATAGTTGCGCCGACAATGACAACAACCATCGCCCACG CATAACCGATATA	60

(*Continued*)

Table 3 List of Backbone Oligonucleotide Sequences (Written From 5′ to 3′), *continued*

Backbone oligonucleotides		
#	Sequence	Length
89	TTCGGTCGCTGAGGCTTGCAGGGAGTTAAAGGCCGCTTTTGCGGGAT CGTCACCCTCAGC	60
90	CTTTTTCATGAGGAAGTTTCCATTAAACGGGTAAAATACGTAATGCC ACTACGAAGGCAC	60
91	CAACCTAAAACGAAAGAGGCAAAAGAATACACTAAAACACTCATCTT TGACCCCCAGCGA	60
92	TTATACCAAGCGCGAAACAAAGTACAACGGAGATTTGTATCATCGCC TGATAAATTGTGT	60
93	CGAAATCCGCGACCTGCTCCATGTTACTTAGCCGGAACGAGGCGCAG ACGGTCAATCATA	60
94	AGGGAACCGAACTGACCAACTTTGAAAGAGGACAGATGAACGGTGTA CAGACCAGGCGCA	60
95	TAGGCTGGCTGACCTTCATCAAGAGTAATCTTGACAAGAACCGGATA TTCATTACCCAAA	60
96	TCAACGTAACAAAGCTGCTCATTCAGTGAATAAGGCTTGCCCTGACG AGAAACACCAGAA	60
97	CGAGTAGTAAATTGGGCTTGAGATGGTTTAATTTCAACTTTAATCAT TGTGAATTACCTT	60
98	ATGCGATTTTAAGAACTGGCTCATTATACCAGTCAGGACGTTGGGAA GAAAAATCTACGT	60
99	TAATAAAACGAACTAACGGAACAACATTATTACAGGTAGAAAGATTC ATCAGTTGAGATT	60
100	TAAGAGCAACACTATCATAACCCTCGTTTACCAGACGACGATAAAAA CCAAAATAGCGAG	60
101	AGGCTTTTGCAAAAGAAGTTTTGCCAGAGGGGGTAATAGTAAAATGT TTAGACTGGATAG	60
102	CGTCCAATACTGCGGAATCGTCATAAATATTCATTGAATCCCCCTCA AATGCTTTAAACA	60
103	GTTCAGAAAACGAGAATGACCATAAATCAAAAATCAGGTCTTTACCC TGACTATTATAGT	60
104	CAGAAGCAAAGCGGATTGCATCAAAAAGATTAAGAGGAAGCCCGAAA GACTTCAAATATC	60
105	GCGTTTTAATTCGAGCTTCAAAGCGAACCAGACCGGAAGCAAACTCC AACAGGTCAGGAT	60
106	TAGAGAGTACCTTTAATTGCTCCTTTTGATAAGAGGTCATTTTTGCG GATGGCTTAGAGC	60
107	TTAATTGCTGAATATAATGCTGTAGCTCAACATGTTTTAAATATGCA ACTAAAGTACGGT	60
108	GTCTGGAAGTTTCATTCCATATAACAGTTGATTCCCAATTCTGCGAA CGAGTAGATTTAG	60
109	TTTGACCATTAGATACATTTCGCAAATGGTCAATAA CCTGTTTAGCTAT	49
110	AACATCCAATAAATCATACAGGCAAGGCAAAGAATTAGCAAAATTAA GCAATAAAGCCTC	60

(*Continued*)

Table 3 List of Backbone Oligonucleotide Sequences (Written From 5′ to 3′), *continued*

Backbone oligonucleotides		
#	Sequence	Length
111	GTGAGCGAGTAACAACCCGTCGGATTCTCCGTGGGAACAAACGGCGG ATTGACCGTAATG	60
112	TTCTTTTCACCAGTGAGACGGGCAACAGCTGATTGCCCTTCACCGCC TGGCCCTGAGAGA	60
113	ATTCGACAACTCGTATTAAATCCTTTGCCCGAACGTTATTAATTTTA AAAGTTTGAGTAA	60
114	TGGGTTATATAACTATATGTAAATGCTGATGCAAATCCAATCGCAAG ACAAAGAACGCGA	60
115	GTTTTAGCGAACCTCCCGACTTGCGGGAGGTTTTGAAGCCTTAAATC AAGATTAGTTGCT	60
116	GTATTAAGAGGCTGAGACTCCTCAAGAGAAGGATTAGGATTAGCGGG GTTTTGCTCAGTA	60
117	AGCGAAAGACAGCATCGGAACGAGGGTAGCAACGGCTACAGAGGCTT TGAGGACTAAAGA	60
118	TAGGAATACCACATTCAACTAATGCAGATACATAACGCCAAAAGGAA TTACGAGGCATAG	60
119	ATTTTCATTTGGGGCGCGAGCTGAAAAGGTGGCATCAATTCTACTAA TAGTAGTAGCATT	60
120	TCTGTCCATCACGCAAATTA	20
121	TATTCATTAAAGGTGAATTA	20

GelRed, 1:300 dilution

1 µl stock GelRed (10,000× in water, Biotium cat. no. 41003)
299 µl 1× PBS
Store up to 1 year at room temperature

Loading dye solution, 6×

1.5 g Ficoll 400 (Biotechnology grade, VWR, cat. no. 89399-840)
1 ml of saturated bromophenol blue (Fisher, cat. no. 115-39-9)
Bring volume to 10 ml with nuclease-free water
Store up to 1 year at room temperature

Composition: 15% Ficoll with bromophenol blue.

$MgCl_2$ stock solution, 300 mM

3 ml 1 M $MgCl_2$ (Alfa Aesar, cat. no. J63150)
7 ml nuclease-free water
Vortex well until the solution is clear
Store up to 6 months at room temperature

PEG solution in 30 mM $MgCl_2$, 4%

4 ml of 10% PEG
1 ml of 300 mM $MgCl_2$
5 ml of nuclease-free water
Vortex well until the solution is clear
Prepare fresh every time before use

Polyethylene glycol (PEG) solution, 10%

1 g of 8000 PEG (ThermoFisher, cat. no. BP233)
Bring volume to 10 ml with nuclease free water
Vortex well until the solution is clear
Store up to 2 weeks at room temperature

Tris-Boric acid-EDTA (TBE), 0.5×

100 ml 5× TBE (see recipe)
900 ml deionized water
Store up to 1 year at room temperature

We recommend filtering the 1× TBE solution to avoid specks on the gel. We use a nylon filtering membrane (0.2 μm pore size, hydrophilic nylon membrane, 47 mm diameter) with a filtration flask. Any 0.2-μm filtration system will probably do the trick.

Tris-Boric acid-EDTA (TBE), 5×

54 g Tris base (VWR, cat. no. 97061-794)
27.5 g boric acid (Millipore Sigma, cat. no. 2710)
20 ml EDTA (0.5 M solution, pH 8; VWR cat. no. BDH7830-1)
Store up to 1 year at room temperature

Contains 445 mM Tris, 445 mM boric acid, and 10 mM EDTA.

COMMENTARY

Background Information

DNA nanotechnology has provided an alternative route to building devices and machines with desired applications (Chandrasekaran, Anderson, Kizer, Halvorsen, & Wang, 2016) including biosensing (Chandrasekaran, Punnoose, et al., 2019). For microRNA detection, different groups have used DNA nanostructures in combination with nanoparticles (Qi et al., 2017; Qu et al., 2017), hybridization chain reaction (Ge et al., 2014), and transition metal dichalcogenide nanosheets (Xiao et al., 2018). Our strategy involves a simple DNA-based device that can be used as a biosensing tool for detecting microRNAs. The DNA nanoswitches have been used in applications such as single-molecule biophysics experiments (Halvorsen, Schaak, & Wong, 2011; Yang, Ward, Halvorsen, & Wong, 2016), quantitative analysis of biomolecular interactions (Koussa, Halvorsen, Ward, & Wong, 2015), and DNA-based computation (Chandrasekaran, Levchenko, Patel, MacIsaac, & Halvorsen, 2017). In the biosensing area, our lab has demonstrated proof-of-concept detection of synthetic DNA sequences (Chandrasekaran, Zavala, & Halvorsen, 2016) and microRNAs (Chandrasekaran, MacIsaac, et al., 2019), while other labs have used the technique for detection of proteins (Hansen, Yang, Koussa, & Wong, 2017). Our assay provides a low-cost approach to microRNA detection, with the ability to be expanded for a range of targets including microRNAs, viral RNAs, long non-coding RNAs, and antigens for different diseases.

Critical Parameters and Troubleshooting

The experiment can be done by almost anyone with basic lab skills. In our lab, we have successfully trained undergraduates in the protocol in as little as a few days. We have additionally run annual hands-on workshops for several years where people have performed a slightly abridged version of this protocol in an 8-hr day.

Through these exercises, we have found a few areas where mistakes are most common. The first and most basic is in making, loading, and running agarose gels. For people new to this, it is worth practicing a few times for loading consistency using DNA ladders. The second area is incomplete linearization of the M13, usually due to incomplete mixing of the enzyme. The third is purification by PEG, which requires a steady hand to avoid accidental loss of nanoswitch material.

Understanding Results

The miRacles assay readout is straightforward for observing the presence of a target microRNA. While running gels for testing, it is suggested to run a control lane with only the total RNA sample. This will show the presence of any genomic DNA and whether this band is similar in migration to the looped band

of the nanoswitch. It would also help in knowing whether the total RNA sample actually has the microRNA of interest, which can be confirmed either through PCR or other control experiments.

Time Considerations

Many steps in this protocol include wait times. The researcher can plan the experiment in such a way that a few steps overlap and are set up during wait times of an earlier step. This will reduce the assay time considerably. In the most extreme case, we previously showed that the assay itself (without nanoswitch construction) could be reduced to 1 hr (Chandrasekaran, MacIsaac, et al., 2019), but the times we report here are recommended to attain the highest sensitivity.

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Literature Cited

Allegra, A., Alonci, A., Campo, S., Penna, G., Petrungaro, A., Gerace, D., & Musolino, C. (2012). Circulating microRNAs: New biomarkers in diagnosis, prognosis and treatment of cancer (Review). *International Journal of Oncology*, *41*(6), 1897–1912. doi: 10.3892/ijo.2012.1647.

Baker, M. (2010). MicroRNA profiling: Separating signal from noise. *Nature Methods*, *7*(9), 687–692. doi: 10.1038/nmeth0910-687.

Bartel, D. P. (2004). MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell*, *116*(2), 281–297. doi: 10.1016/S0092-8674(04)00045-5.

Chandrasekaran, A. R. (2019). Detecting miRNAs using DNA nanoswitches. *Trends in Biochemical Sciences*, *44*(9), 819–820. doi: 10.1016/j.tibs.2019.06.005.

Chandrasekaran, A. R., Anderson, N., Kizer, M., Halvorsen, K., & Wang, X. (2016). Beyond the fold: Emerging biological applications of DNA origami. *ChemBioChem*, *17*(12), 1081–1089. doi: 10.1002/cbic.201600038.

Chandrasekaran, A. R., Levchenko, O., Patel, D. S., MacIsaac, M., & Halvorsen, K. (2017). Addressable configurations of DNA nanostructures for rewritable memory. *Nucleic Acids Research*, *45*(19), 11459–11465. doi: 10.1093/nar/gkx777.

Chandrasekaran, A. R., MacIsaac, M., Dey, P., Levchenko, O., Zhou, L., Andres, M., … Halvorsen, K. (2019). Cellular microRNA detection with miRacles: MicroRNA- activated conditional looping of engineered switches. *Science Advances*, *5*(3), eaau9443. doi: 10.1126/sciadv.aau9443.

Chandrasekaran, A. R., Punnoose, J. A., Zhou, L., Dey, P., Dey, B. K., & Halvorsen, K. (2019). DNA nanotechnology approaches for microRNA detection and diagnosis. *Nucleic Acids Research*, *47*(20), 10489–10505. doi: 10.1093/nar/gkz580.

Chandrasekaran, A. R., Zavala, J., & Halvorsen, K. (2016). Programmable DNA nanoswitches for detection of nucleic acid sequences. *ACS Sensors*, *1*(2), 120–123. doi: 10.1021/acssensors.5b00178.

Cortez, M. A., Bueso-Ramos, C., Ferdin, J., Lopez-Berestein, G., Sood, A. K., & Calin, G. A. (2011). MicroRNAs in body fluids—The mix of hormones and biomarkers. *Nature Reviews Clinical Oncology*, *8*(8), 467–477. doi: 10.1038/nrclinonc.2011.76.

Dong, H., Lei, J., Ding, L., Wen, Y., Ju, H., & Zhang, X. (2013). MicroRNA: Function, detection, and bioanalysis. *Chemical Reviews*, *113*(8), 6207–6233. doi: 10.1021/cr300362f.

Ebert, M. S., & Sharp, P. A. (2012). Roles for MicroRNAs in conferring robustness to biological processes. *Cell*, *149*(3), 515–524. doi: 10.1016/j.cell.2012.04.005.

Friedman, R. C., Farh, K. K.-H., Burge, C. B., & Bartel, D. P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome Research*, *19*(1), 92–105. doi: 10.1101/gr.082701.108.

Ge, Z., Lin, M., Wang, P., Pei, H., Yan, J., Shi, J., … Zuo, X. (2014). Hybridization chain reaction amplification of MicroRNA detection with a tetrahedral DNA nanostructure-based electrochemical biosensor. *Analytical Chemistry*, *86*(4), 2124–2130. doi: 10.1021/ac4037262.

Halvorsen, K., Kizer, M. E., Wang, X., Chandrasekaran, A. R., & Basanta-Sanchez, M. (2017). Shear dependent LC purification of an engineered DNA nanoswitch and implications for DNA origami. *Analytical Chemistry*, *89*(11), 5673–5677. doi: 10.1021/acs.analchem.7b00791.

Halvorsen, K., Schaak, D., & Wong, W. P. (2011). Nanoengineering a single-molecule mechanical switch using DNA self-assembly. *Nanotechnology*, *22*(49), 494005. doi: 10.1088/0957-4484/22/49/494005.

Hansen, C. H., Yang, D., Koussa, M. A., & Wong, W. P. (2017). Nanoswitch-linked immunosorbent assay (NLISA) for fast, sensitive, and specific protein detection. *Proceedings of the National Academy of Sciences*, *114* (39), 10367–10372. doi: 10.1073/pnas.1708148114.

Hunt, E. A., Broyles, D., Head, T., & Deo, S. K. (2015). MicroRNA detection: Current technology and research strategies. *Annual Review of Analytical Chemistry*, *8*(1), 217–237. doi: 10.1146/annurev-anchem-071114-040343.

Koussa, M. A., Halvorsen, K., Ward, A., & Wong, W. P. (2015). DNA nanoswitches: A quantitative platform for gel-based biomolecular interaction analysis. *Nature Methods*, *12*(2), 123–126. doi: 10.1038/nmeth.3209.

Koussa, M. A., Sotomayor, M., & Wong, W. P. (2014). Protocol for sortase-mediated construction of DNA—protein hybrids and functional nanostructures. *Methods*, *67*(2), 134–141. doi: 10.1016/j.ymeth.2014.02.020.

Lee, R. C., Feinbaum, R. L., & Ambros, V. (1993). The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*, *75*(5), 843–854. doi: 10.1016/0092-8674(93)90529-Y.

Qi, L., Xiao, M., Wang, X., Wang, C., Wang, L., Song, S., … Pei, H. (2017). DNA-encoded Raman-active anisotropic nanoparticles for microRNA detection. *Analytical Chemistry*, *89*(18), 9850–9856. doi: 10.1021/acs.analchem.7b01861.

Qu, X., Zhu, D., Yao, G., Su, S., Chao, J., Liu, H., … Fan, C. (2017). An exonuclease III-powered, on-particle stochastic DNA walker. *Angewandte Chemie International Edition*, *56*(7), 1855–1858. doi: 10.1002/anie.201611777.

Rothemund, P. W. K. (2006). Folding DNA to create nanoscale shapes and patterns. *Nature*, *440*(7082), 297–302. doi: 10.1038/nature04586.

Ryan, B. M., Robles, A. I., & Harris, C. C. (2010). Genetic variation in microRNA networks: The implications for cancer research. *Nature Reviews Cancer*, *10*(6), 389–402. doi: 10.1038/nrc2867.

Vidigal, J. A., & Ventura, A. (2015). The biological functions of miRNAs: Lessons from in vivo studies. *Trends in Cell Biology*, *25*(3), 137–147. doi: 10.1016/j.tcb.2014.11.004.

Weber, J. A., Baxter, D. H., Zhang, S., Huang, D. Y., Huang, K. H., Lee, M. J., … Wang, K. (2010). The MicroRNA spectrum in 12 body fluids. *Clinical Chemistry*, *56*(11), 1733–1741. doi: 10.1373/clinchem.2010.147405.

Xiao, M., Chandrasekaran, A. R., Ji, W., Li, F., Man, T., Zhu, C., … Li, L. (2018). Affinity-modulated molecular beacons on MoS2 nanosheets for MicroRNA detection. *ACS Applied Materials & Interfaces*, *10*(42), 35794–35800. doi: 10.1021/acsami.8b14035.

Yang, D., Ward, A., Halvorsen, K., & Wong, W. P. (2016). Multiplexed single-molecule force spectroscopy using a centrifuge. *Nature Communications*, *7*, 11026. doi: 10.1038/ncomms11026.

Zhang, L., Chen, X., Su, T., Li, H., Huang, Q., Wu, D., … Han, Z. (2015). Circulating miR-499 are novel and sensitive biomarker of acute myocardial infarction. *Journal of Thoracic Disease*, *7*(3), 303–308.