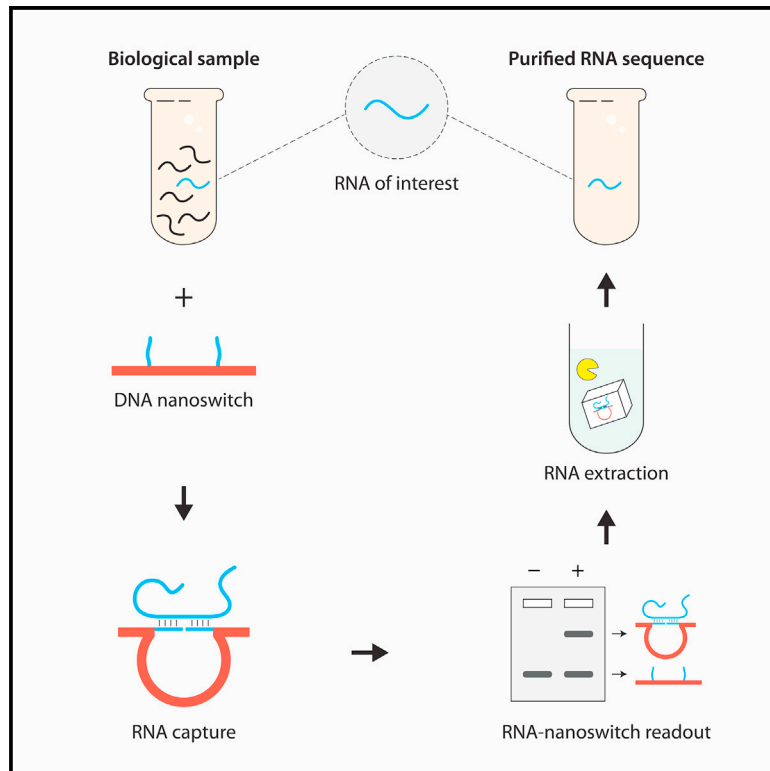


# Sequence-selective purification of biological RNAs using DNA nanoswitches

## Graphical abstract



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## In brief

Zhou et al. present a low-cost, benchtop method using DNA-based nanoswitches to purify specific RNA sequences from biological extracts. RNAs from 22 to 401 nt are purified with high recovery and purity, competitive with beads-based methods. The method can be paired with LC/MS for identifying RNA modifications in specific RNAs.

## Highlights

- DNA nanoswitch technology enables benchtop purification of specific RNA sequences
- RNAs from 22 to 401 nt are purified with up to 75% recovery and 99.98% purity
- DNA nanotechnology approach enables multiplexing and plug-and-play versatility
- Can be paired with downstream LC/MS for finding RNA modifications in specific RNAs



## Article

## Sequence-selective purification of biological RNAs using DNA nanoswitches

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**MOTIVATION** Purification of RNA is a major aspect of RNA biology. In many instances, purification can pose a large challenge and expense, and few methods exist for the selective purification of individual RNAs by sequence. As tools in RNA biology improve to determine structures, chemical modifications, and interactions of individual RNAs, so too must preparative tools improve to allow purification of individual RNAs. Our approach to this problem enables sequence-selective purification of RNAs, and also paves the way to more difficult purification of RNA complexes.

## SUMMARY

Nucleic acid purification is a critical aspect of biomedical research and a multibillion-dollar industry. Here we establish sequence-selective RNA capture, release, and isolation using conformationally responsive DNA nanoswitches. We validate purification of specific RNAs ranging in size from 22 to 401 nt with up to 75% recovery and 99.98% purity in a benchtop process with minimal expense and equipment. Our method compared favorably with bead-based extraction of an endogenous microRNA from cellular total RNA, and can be programmed for multiplexed purification of multiple individual RNA targets from one sample. Coupling our approach with downstream LC/MS, we analyzed RNA modifications in 5.8S ribosomal RNA, and found 2'-O-methylguanosine, 2'-O-methyluridine, and pseudouridine in a ratio of ~1:7:22. The simplicity, low cost, and low sample requirements of our method make it suitable for easy adoption, and the versatility of the approach provides opportunities to expand the strategy to other biomolecules.

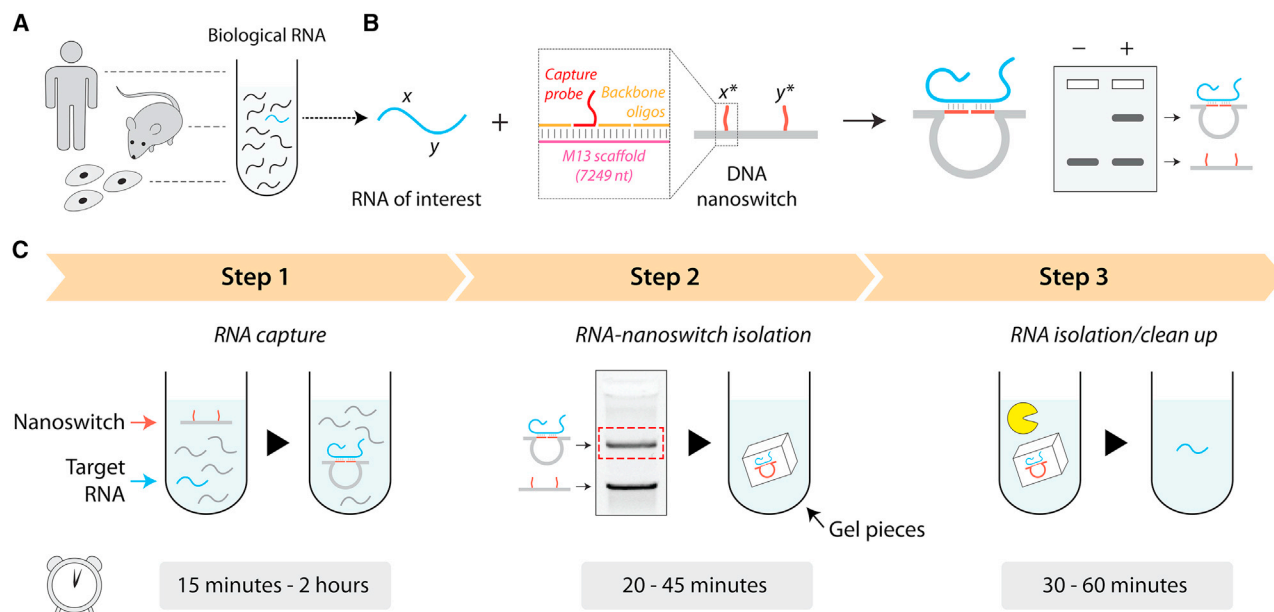
## INTRODUCTION

Purification is a cornerstone of RNA research, arguably beginning in 1868 when Friedrich Miescher achieved the first nucleic acid ("nuclein") purification (Dahm, 2005). Since then, many types of RNA with diverse functions have been discovered, including messenger RNA (mRNA), catalytic ribozymes, self-splicing RNAs, and gene regulating RNAs (Elliott and Ladomery, 2017). The importance of RNA in human health is now well appreciated, especially with many viruses, including SARS-CoV-2 having RNA as their genetic information carrier. In addition, discoveries of microRNAs, long noncoding RNAs, and chemically modified RNAs have reshaped our understanding of the importance of RNA in biological processes and diseases (Esteller, 2011; Manoharan, 2004; Roundtree et al., 2017).

Most RNA research depends on high-quality RNA that is free from contaminants such as genomic DNA, enzymes, and organic

solvents. For this reason, RNA purification has become a multi-billion-dollar business that is still growing. Most methods isolate all or a subset of RNAs (e.g., mRNAs, small RNAs) from a biological sample and are commonly done with gel electrophoresis (Lukavsky and Puglisi, 2004), liquid chromatography (Waghmare et al., 2009), organic extraction (Rio et al., 2010), or spin columns (Shi et al., 2018). Beyond this, there is a need for purifying specific RNA sequences for direct analysis of RNA structure and function. For example, purified RNA sequences are required to study RNA modifications within particular sequences with liquid chromatography/mass spectrometry (LC/MS) methods, or to study biomolecular interactions of RNA with single-molecule methods. However, purification of specific RNAs is substantially more difficult and expensive. Magnetic beads with single-stranded DNA (ssDNA) capture probes is currently the only major approach for performing such isolations (Adams et al., 2015; Benrseimer, 2006). Commercially available bead-based kits purify





**Figure 1. DNA nanoswitch overview and workflow of purification**

(A) Challenge of single species RNA purification.

(B) The DNA nanoswitch converts from a linear to looped form in the presence of target RNA.

(C) The purification workflow consists of three major steps: RNA capture, RNA-nanoswitch isolation, and final RNA cleanup.

panels or subtypes of RNAs (e.g., microRNA panels or poly-adenylated mRNAs). Purifying an individual RNA sequence typically requires customizing the beads, and multiplexed purification of RNA sequences from a single sample is not possible.

Here we developed a novel catch-and-release strategy for targeted purification of unique RNA sequences using conformationally responsive nanoswitches constructed from DNA. DNA has been previously used as a building material for the creation of rigid and dynamic nanostructures with prescribed geometries and functions (Jones et al., 2015; Xavier and Chandrasekaran, 2018). Some dynamic DNA structures, including our nanoswitches, undergo a conformational change in response to external molecules or environment, for applications, including biosensing (Xiao et al., 2019), imaging (Chakraborty et al., 2016), molecular computation (Scalise and Schulman, 2019), biomolecular analysis (Lee and Wälti, 2019), and drug delivery (Madhanagopal et al., 2018). Here we demonstrate the first use of a DNA nanotechnology-based approach for RNA purification. We show that our new technique is capable of specific RNA purification with near-complete depletion of nonspecific RNAs and sufficient recovery yield for downstream applications such as mass spectrometry to identify the presence of RNA modifications.

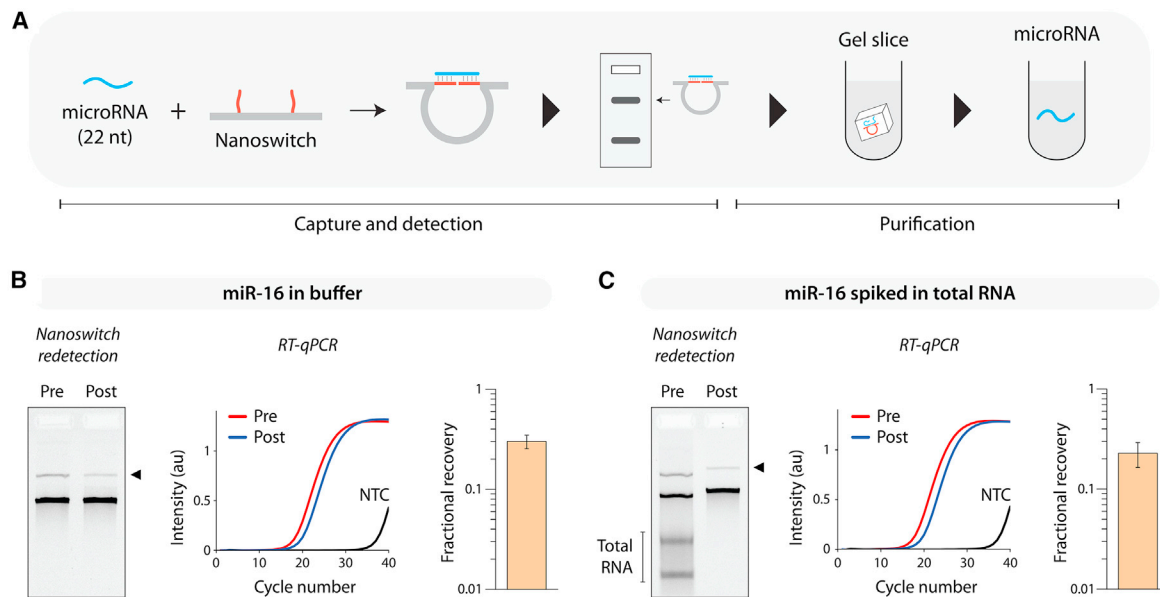
This work develops and validates a DNA nanoswitch assay for capture and extraction of target RNA molecules (Figure 1A). The DNA nanoswitch is designed and constructed based on the principles of DNA origami (Rothemund, 2006), by hybridizing an ssDNA scaffold (M13 viral genome, 7,249 nucleotides) with a set of oligonucleotides to make the nanoswitch entirely double stranded (Figure S1) (Chandrasekaran et al., 2016; Koussa et al., 2015; Zhou et al., 2020). Two of these oligonucleotides

are modified to contain ssDNA capture probes that can recognize the RNA sequence of interest. Capture of the target RNA causes the nanoswitch to reconfigure to a looped state (Figure 1B) (Chandrasekaran et al., 2019). The looped and unlooped conformations of the nanoswitch migrate differently on an agarose gel during electrophoresis, providing a unique signal that indicates capture of the target RNA.

To use DNA nanoswitches for purification of RNAs, we developed a general strategy that consists of three steps: (1) RNA capture by DNA nanoswitches, (2) isolation of the RNA-nanoswitch complexes, and (3) isolation and cleanup of the RNA, yielding pure RNA target molecule (Figure 1C). The first step consists of mixing the DNA nanoswitches with the solution containing target RNA molecules, causing the nanoswitches to become looped. The second step is to separate the looped nanoswitches using gel electrophoresis and to excise the gel piece containing the looped nanoswitches. The third step requires removal of the RNA from the nanoswitch and from the gel.

## RESULTS

To accomplish nanoswitch-based purification, our main challenges were ensuring high-efficiency RNA capture with DNA nanoswitches, and establishing a workflow for downstream processing of the RNA-nanoswitch complex. To demonstrate proof-of-concept for our sequence-selective purification, we chose a synthetic microRNA (22 nt). In our previous work on microRNA detection, we used capture probes that were typically 11 nt long and demonstrated high-efficiency sub-picomolar detection ability (Chandrasekaran et al., 2019). Here, we adopted a similar nanoswitch design for the detection and purification of our target



**Figure 2. Validation of RNA purification**

(A) Overview of entire purification process.

(B) Validation and quantification of the recovery for miR-16 in buffer, compared with no template control (NTC).

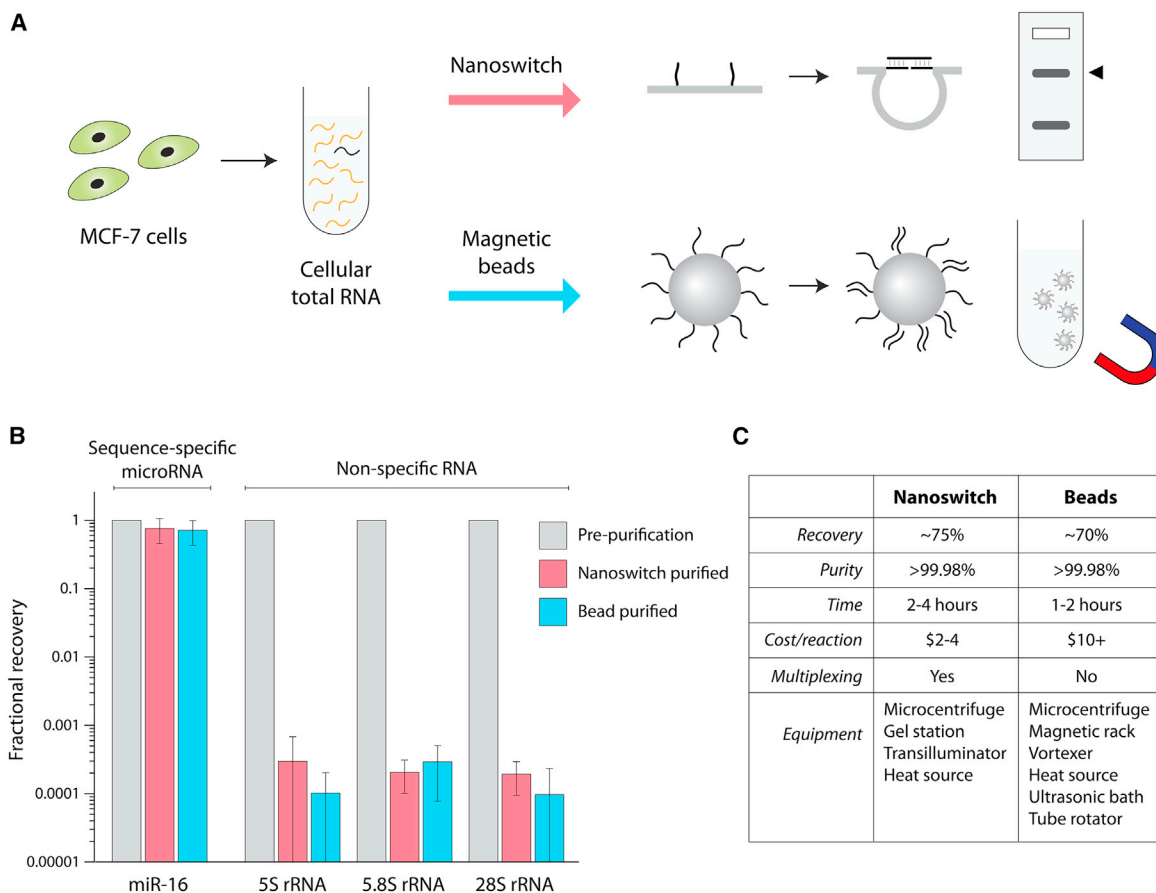
(C) Validation and quantification of the recovery for miR-16 spiked into total RNA. Bar graphs show mean and standard deviation from three purification replicates.

microRNA, miR-16. With nanoswitches developed to efficiently capture the target RNA and report the capture by gel electrophoresis, we turned to downstream processing of the RNA-nanoswitch complex. This first requires excision of the agarose gel slice containing the RNA-nanoswitch complex followed by separation of the captured RNA from both the nanoswitch and the agarose gel matrix. To remove the nanoswitch, we chose digestion by DNase I, which is known to efficiently digest double-stranded DNA as well as ssDNA either alone or in a DNA-RNA hybrid (Suck, 1994). We found that DNase I effectively digests the nanoswitch even when it is embedded in agarose (Figure S2), enabling release of the RNA. Following gel excision and DNase I digestion, we were left with our target RNA, DNA nucleotides or small fragments, and agarose gel pieces. To isolate the RNA, we tested variations of a commercial RNA gel extraction kit and crush-and-soak extraction followed by column purification to remove nucleotides and enzymes (Figure S3). While all of these downstream methods worked to various degrees, we found the highest recovery of the microRNA using a crush-and-soak protocol with an oligo cleanup kit. For maximum recovery, we also replaced our typical UV-excited dye (GelRed) with a blue light excited dye (EvaGreen) to avoid UV damage to RNA (Figure S3).

To validate the purification and quantify recovery of our target RNA, we reacted the synthetic microRNA target with a molar excess of DNA nanoswitches for 2 h, and then ran gels, excised the looped bands, and processed the material using our developed protocol (Figure 2A). To confirm purification of the correct sequence, we show both “before and after” detection of the purified product with the nanoswitches as well as with RT-qPCR. Based on these results, we had a mean recovery of 30% for

miR-16 (Figure 2B). Next, we spiked the miR-16 into 250 ng total RNA to mimic a biological context and again validated purification and recovery. In the presence of total RNA, we observed 23% recovery of miR-16 (Figure 2C), with an observable elimination of off-target RNA (gel in Figure 2C).

After validating our method with synthetic spike-ins, we moved to apply our purification to an endogenous RNA. To achieve this, we selected miR-16 as the target RNA, which is a known cancer biomarker, with roles in apoptosis of cancer cells, suppression of tumor invasiveness, cell migration, and tumor progression (Aqeilan et al., 2010; Liang et al., 2015). As the model biological sample, we chose total RNA from MCF-7 cells, a known cancerous cell line (Horwitz et al., 1975). We first tested the nanoswitches with 250 ng of MCF-7 total RNA to confirm that we can capture endogenous miR-16 (Figure S3). To purify endogenous miR-16, we incubated MCF-7 total RNA with DNA nanoswitches for 2 h and performed the purification protocol we optimized for microRNAs (Figure 3A). We found that we were able to purify miR-16 from total RNA with an apparent efficiency of ~75%, based on RT-qPCR comparing levels of miR-16 in 250 ng of total RNA to the miR-16 in our purified product. To confirm the purity of the sample, we also probed three different rRNAs (5S, 5.8S, and 28S rRNAs). Together these rRNAs comprise a majority of cellular RNA, and represent the most likely nonspecific contaminant in purification due to their abundance. We found that ~99.98% of those rRNAs were removed (Figure 3B), illustrating the specificity of our approach. Next, we compared the performance of our strategy with a commercially available beads-based purification method (Figure 3B). Following the manufacturer’s protocol, we found that the beads had nearly identical performance characteristics (within



**Figure 3. Purification of endogenous miR-16 from total RNA**

(A) Illustration showing purification of miR-16 from cellular total RNA using nanoswitch method and commercially available beads-based method.

(B) Recovery of miR-16 from MCF-7 total RNA and elimination of nonspecific rRNAs using DNA nanoswitches and beads-based method. Purification was performed in duplicate for miR-16 measurement and triplicate for nonspecific RNA, with bars representing mean and standard deviation. Non-amplifying wells were excluded from analysis.

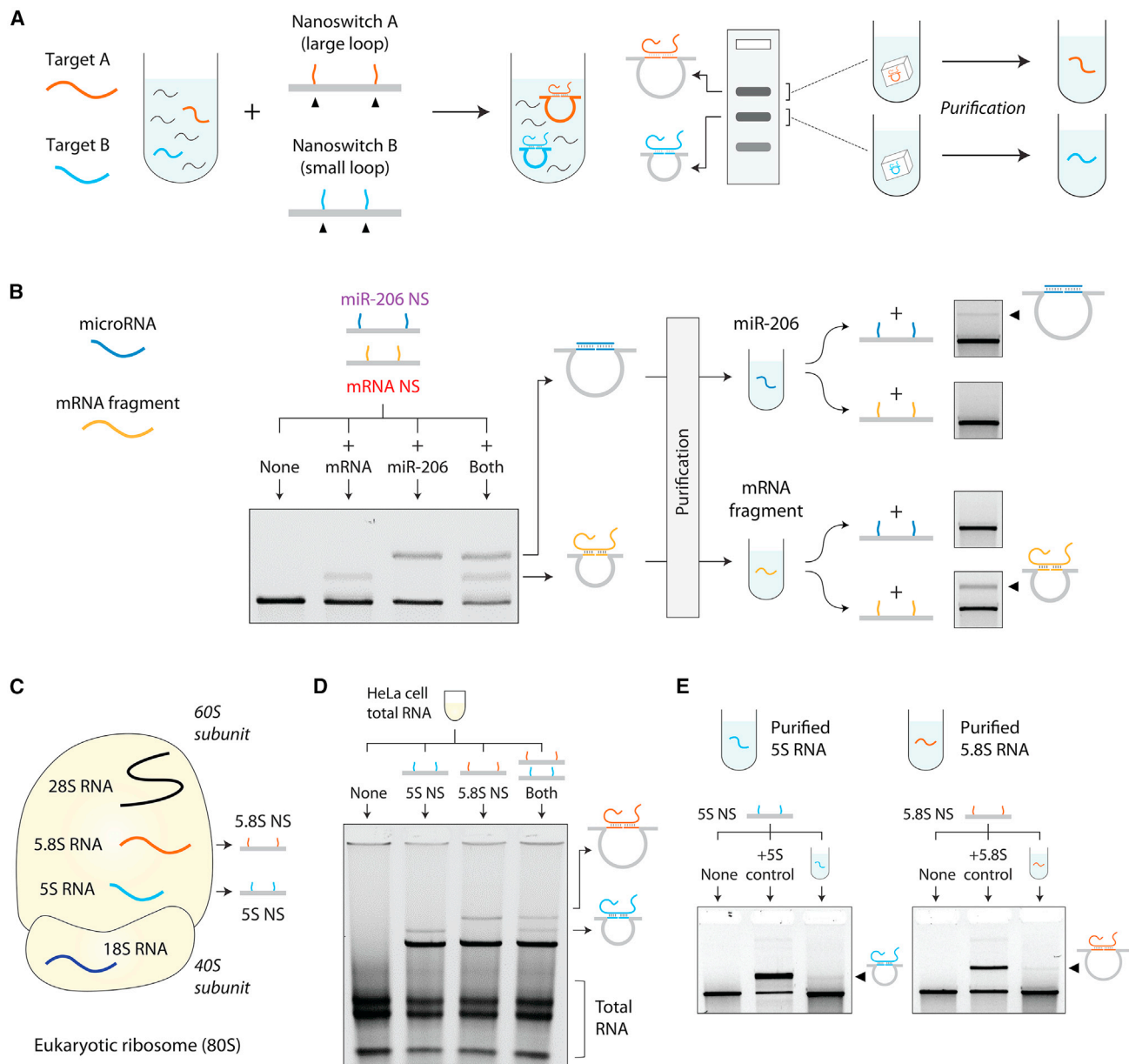
(C) Comparison of DNA nanoswitch method with commercial beads-based method.

uncertainty). These data show that this first proof-of-concept of our method is already performing similar to commercial solutions (Figure 3C).

After validating the method for microRNAs, we tested purification of a longer mRNA fragment (401 nt). As a model mRNA, we used a 401-nt transcribed mRNA fragment from the 3' UTR of a *Drosophila* mRNA (Flora et al., 2018). We chose a 60-nt target region within the mRNA sequence and tested different capture probe lengths (15, 20, 30 nt) and found that the nanoswitch with 30 nt was the most efficient (Figure S3). For purification, we incubated the mRNA fragment with DNA nanoswitches and ran gels, excised bands, and processed the same as mentioned above for microRNAs, but with specific modifications for the longer RNA fragment. We found a mean recovery of ~5% for the mRNA (Figure S3). We noticed that the mRNA fragment has a lower apparent capture efficiency (possibly due to secondary structure in the RNA), which seems to account for most of the recovery difference when compared with microRNA.

A unique feature of the DNA nanoswitches is that they can be programmed to enable multiplexing (Chandrasekaran et al.,

2019), which we used here for simultaneous purification of different RNA molecules from a single reaction (Figure 4A). This cannot be easily accomplished by other methods, such as beads-based purification, which are limited to either purifying a cohort of molecules together, or in running separate reactions to recover different molecules separately. To achieve multiplexing, we redesigned the location of the capture probes to create two nanoswitches with different loop sizes. The mRNA nanoswitch was designed to form a smaller loop with faster migration, while a microRNA nanoswitch was designed to form a larger loop with slower migration (Figures 4B and S4). On capturing the specific target RNA molecules, the looped nanoswitches can be identified by unique bands on a gel, allowing excision of the bands corresponding to specific target RNAs. With this design, we showed detection and individual purification of miR-206 and 401-nt mRNA fragment at the same time (Figure 4B). Redetection of the purified products with nanoswitches demonstrated the specificity of the purification method. Each nanoswitch only detected the correct purified target (miR-206 or mRNA), indicating successful isolation of target RNA without notable



**Figure 4. Multiplexed purification: concept, validation, and application to rRNAs**

(A) Concept of multiplexed purification.

(B) Validation of multiplexed purification using an mRNA fragment and a microRNA.

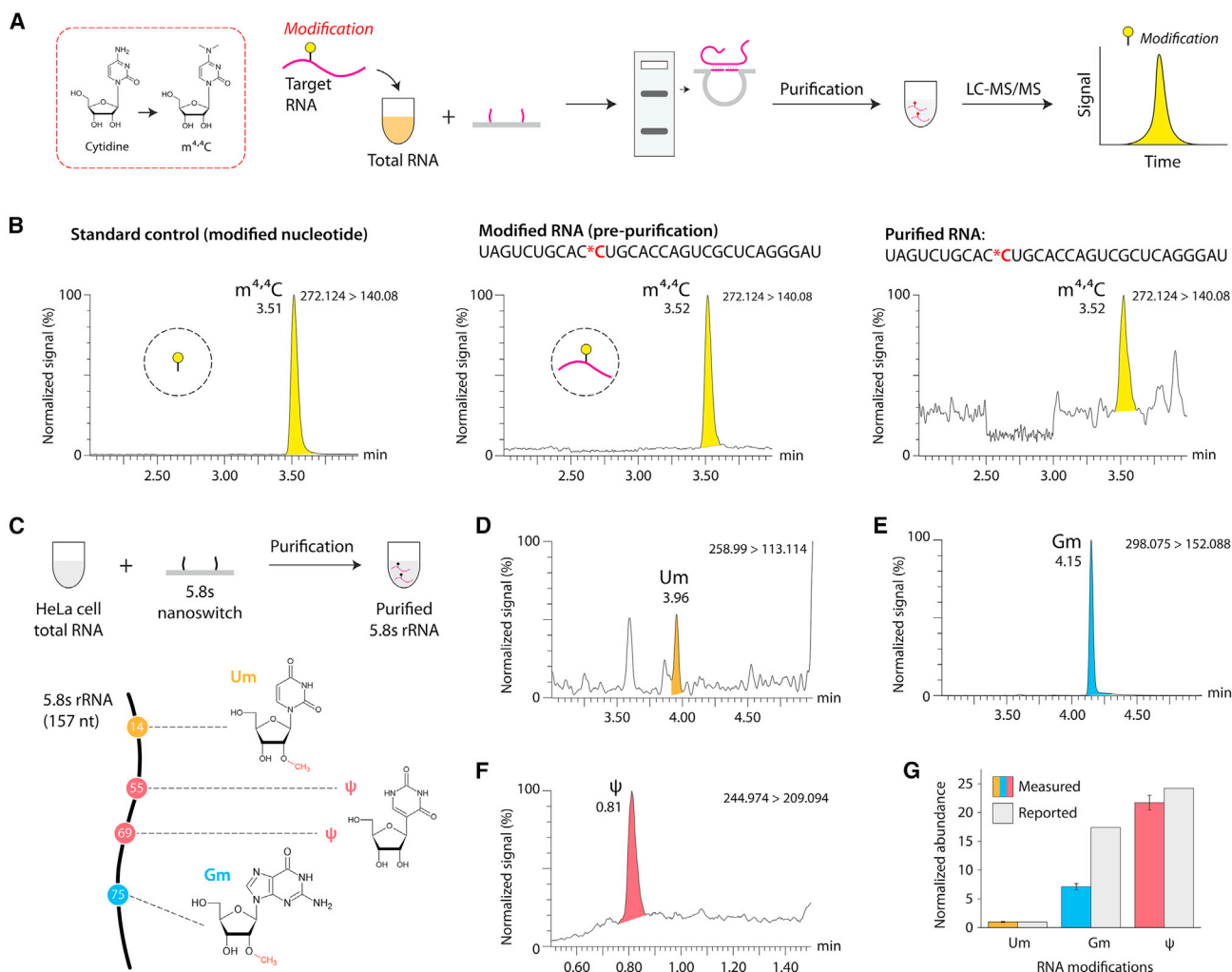
(C and D) Application of multiplexed purification in selecting 5S and 5.8S rRNA from HeLa cell total RNA.

(E) Validation of purified rRNAs by re-detecting with nanoswitches.

cross-contamination (Figure 4B and Figure S4). Our previous work has demonstrated multiplexed detection of up to six different DNA fragments, suggesting that multiplexed purification could be similarly expanded (Chandrasekaran et al., 2021).

We further extended this multiplexing to purify multiple RNAs from real biological samples. We developed multiplexed nanoswitches targeting the 5.8S and 5S rRNAs from HeLa cell total RNA (Figure 4C). The 5.8S and 5S subunits (~160 and ~120 nt, respectively) are critical for protein translation (Gillespie

et al., 2006), and have been shown to contain chemical modifications including pseudouridine (Decatur and Schnare, 2008; Taoka et al., 2018). We chose 30-nt target regions for each based on secondary structure analysis and designed nanoswitches to form a large loop for 5.8S rRNA and a small loop for 5S rRNA (Figure S4). We showed clear multiplexed capture of the 5.8S and 5S rRNAs directly from 250 ng total RNA from HeLa cells (Figure 4D). For multiplexed purification, we captured both rRNAs in a single reaction from 2 μg total RNA of HeLa cell



**Figure 5. Purification of RNA with chemical modification**

(A) The process of purification and verification by LC-MS/MS of modified RNAs.

(B) Modification probing of the purified RNA molecules by LC-MS/MS. Left: m<sup>4,4</sup>C modification standard, middle: synthetic RNA with modification, and right: purified RNA from 10 nM sample. The target RNA molecule has an m<sup>4,4</sup>C modification on a cytosine (sequence shown above graph on right, \* indicates position of modification).

(C) Purification of 5.8S rRNA from HeLa cell total RNA and illustration of known modifications and their positions.

(D–F) Probing 2'-O-methyluridine (Um), 2'-O-methylguanosine (Gm), and pseudouridine (ψ) modifications in the purified 5.8S rRNA by LC-MS/MS and (G) mole ratio of the three modifications Um, Gm, and ψ. Bars represent mean and standard deviation from two technical replicates of a single purified sample.

and purified each rRNA species (Figure S4), and confirmed successful purification of the correct rRNA in each instance (Figure 4E).

One compelling application of our approach is to study how the >100 natural RNA modifications can alter biological functions of particular RNAs (Boccaletto et al., 2018). For example, N6-methyladenosine (m<sup>6</sup>A) affects the stability of mRNA and protein translation (Wang et al., 2014), rRNA modifications can influence translation efficiency (Sloan et al., 2016), and some RNA modifications appear in response to viral infection (McIntyre et al., 2018). One of the gold standard methods for measuring RNA modifications is ultra-high performance LC-tandem MS (UHPLC-MS/MS) (Basanta-Sanchez et al., 2016), but this

method typically uses digested RNA and loses sequence information. Used in conjunction with our purification method, RNA modifications can be measured on specific RNA sequences (Figure 5A).

To test this application, we chose the m<sup>4,4</sup>C (N<sup>4</sup>, N<sup>4</sup>-dimethylcytosine) modification and synthesized a short (31 nt) RNA with a single m<sup>4,4</sup>C modification (Figure 5B). The m<sup>4,4</sup>C modification has relevance in viral infection (Mao et al., 2020; McIntyre et al., 2018), and to our knowledge can only be identified by MS methods. To mimic biological samples, we spiked the modified RNA at different concentrations (10 nM and 1 nM) into 2 μg of total RNA from HeLa cells and performed the purification (Figure S5). We showed detection of the modification from the

purified samples at both concentrations, demonstrating that our method can be applied to extract target RNA with chemical modifications with enough material for downstream UHPLC-MS/MS testing (Figures 5B and S5). To expand on this capability, we looked for RNA modifications in 5.8S rRNA, which is known to have four nucleotides with three different modifications: 2'-O-methyluridine (Um), 2'-O-methylguanosine (Gm), and pseudouridine ( $\psi$ ) (Figure 5C) (Taoka et al., 2018). We purified several gel lanes of 5.8S rRNA, and processed samples for UHPLC-MS/MS. We found evidence of all three of these modifications in our purified rRNA sample (Figures 5D–5F), including Um, which was previously reported as only being modified in 5% of 5.8S rRNAs at a single base position. Our analysis yielded a relative mole ratio of ~1:7:22 for Um:Gm: $\psi$ , while a previous report predicted a 1:17:24 relative mole ratio (Figure 5G) (Taoka et al., 2018). Our analyses produced the same overall trend of modifications, with the biggest discrepancy being that Gm in our results appears about half as frequently as expected.

### DISCUSSION

In summary, we present a novel catch-and-release method for sequence-selective RNA purification using DNA nanoswitches. Building on the success of high detection sensitivity of nucleic acids (Chandrasekaran et al., 2019), we have shown that this detection step can be coupled with simple downstream extraction and cleanup steps to yield purified RNA in around 2–4 h. As a proof-of-concept, we demonstrated that our method could purify various RNAs, including mRNA fragments, microRNAs, rRNAs, and chemically modified RNAs spanning length scales from tens to hundreds of nucleotides.

Our approach provides both new purification capabilities as well as technical advantages over some existing purification methods. The capability to perform multiplexed purifications and the feature of detecting the purification target as part of the process are unique to our method. The capture probes of our nanoswitches can be readily programmed for multiple purification targets, either separately or in parallel to enable purification of five or more RNA molecules in a single reaction. In comparing with affinity capture using beads, our approach has a few technical advantages. First, the DNA nanoswitch avoids surface binding, which can cause nonspecific interactions with off-targets and potentially decrease purity. Second, our method provides visual pre-detection of the target RNA during the purification process, aiding in troubleshooting and yield estimation. These features may be especially important for longer RNAs where beads require modification with customized probes (unlike commercially available microRNA beads). In these cases, binding and elution protocols need to be potentially altered and validated for each target, without feedback until the endpoint of the purification assay. Our method can also detect and purify rare RNA molecules in a limited amount of sample due to the demonstrated attomole detection ability of the DNA nanoswitches (Chandrasekaran et al., 2019). The nanoswitches also provide a quick, affordable, do-it-yourself alternative to purifying individual RNAs that can be performed at low cost on the benchtop (Table S1). Our nanoswitches cost less than a penny per reaction, and can be easily designed and constructed for

purifying only the desired RNAs. To offset the initial cost for labs that want to try the DNA nanoswitch method, our lab is willing to provide sample aliquots of the backbone oligonucleotide mix for nonprofit research purposes on reasonable request.

Our approach also has some limitations that leave room for improvement in future development. These include the relatively small scale of purification and manual processing of samples. The purification scale is currently limited by the amount of nanoswitch and its efficient isolation. With nanoswitches at nanomolar concentrations and microliter volumes, our approach currently purifies RNA molecules at the femtomole scale. Scaling up volumes and processing many gel lanes could likely get to picomole scale purification. Alternative separation technologies, such as LC, could potentially push the purification scale even higher (Halvorsen et al., 2017). Similarly, LC or other techniques, like the BluePippin gel cassette (Durin et al., 2012), could help reduce manual processing and even facilitate automated purification. Overall, we expect that future improvements could increase overall yield and purification scale, expand multiplexing, and further reduce the cost and time for processing. Beyond this proof-of-concept validation, our method is also compatible with the many upgrades that current techniques such as beads-based purification use. For example, the capture probes of the DNA nanoswitch can be modified to contain locked nucleic acids (Jacobsen et al., 2011) or morpholino bases (Wages et al., 1997) that are known to improve capture efficiency.

Our method is well suited to purify individual RNA species from a large pool. This is in contrast to beads-based methods where commercial kits contain probes for RNA panels (for microRNAs) or for classes of RNAs (e.g., mRNA or rRNA). Other affinity-based methods may be well suited for separating synthesized products from impurities (Batey and Kieft, 2007; Karlsson et al., 2020; Kieft and Batey, 2004; McCarthy et al., 2009), but are not easily adaptable to purify any RNA target sequence from cellular RNA. Purifying biological RNAs is necessary for many downstream applications, including sequencing, structural imaging, biomolecular interaction analysis, RNA modification analysis, and single-molecule experimentation. Analysis of chemical modifications of RNA is a particularly attractive application since MS techniques can detect low levels of modifications but tend to lose sequence information. By purifying single RNA species for MS (Basanta-Sanchez et al., 2016), cryo-electron microscopy analysis (Natchiar et al., 2017), or epitranscriptome sequencing technologies (Li et al., 2017), it will be easier to determine which particular RNAs contain modifications.

Beyond RNA, our general strategy is likely to work with minor modifications for the purification of other biomolecules for which affinity reagents are available. Detection of proteins, including antibodies, has been demonstrated previously (Koussa et al., 2015), and purification with this strategy should be relatively straightforward. More advanced purifications of molecular complexes such as ribonucleoproteins may also be possible by targeting either one or both molecular components with the DNA nanoswitches. The versatility of the approach lends itself to many different applications. It can be seen from the history of scientific literature that advances in purification tend to precede new discoveries (e.g., Dr. Miescher's isolation of DNA in 1868).



We hope that our approach will similarly facilitate new discoveries, both in RNA science and beyond.

### Limitations of the study

Favorable outcomes of our method rely on DNA nanoswitches that are properly constructed and purified from excess oligos. Nanoswitches should be tested for proper activity using a DNA or RNA target oligo before use. Incubation temperature and reaction time may need to be altered based on the overall length of the target and the length of the two “capture” regions in the nanoswitch. RNA targets longer than those attempted here may be possible, but secondary structures could inhibit binding. All materials should be free from nucleases to avoid degradation of either the DNA nanoswitches or the RNA target – this includes the often overlooked gel imaging surface and cutting tool to excise bands. For biologically derived or *in vitro* transcribed RNA samples, we recommend pre-processing samples to remove DNases or eliminate DNase activity, either through a column-based removal or by heat inactivation. Choice of gel stain and gel staining procedures can alter performance of the assay – in particular GelRed is known to stabilize duplexes, so prestaining and poststaining can cause differences. Different gel stains may also result in altered migration patterns of nanoswitches, and may have variability in their removal after purification. Most column-based cleanup kits are known to remove dyes including GelRed, but applications involving other dyes or sensitive downstream applications may require further validation to ensure a dye-free product.

### STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- METHOD DETAILS
  - DNA nanoswitches
  - RNA samples
  - Nanoswitch detection assays
  - RNA purification protocol
  - Modification analysis by LC-MS
  - RT-qPCR assays
  - Beads purification
- QUANTIFICATION AND STATISTICAL ANALYSIS

### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.crmeth.2021.100126>.

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### AUTHOR CONTRIBUTIONS

L.Z., A.R.C., and K.H. designed the major experiments. L.Z., A. H., J.V., and C.C. performed nanoswitch experiments. A.H., J.V., L.Z., P.D., and B.K.D. designed and performed qRT-PCR experiments. S.M. and J.S. designed and synthesized the modified oligonucleotide. P.R. provided materials and training for mRNA fragment transcription. L.Z. wrote the first draft of the manuscript. L.Z., A.R.C., and K.H. co-wrote later drafts of the manuscript with comments from other authors.

### DECLARATION OF INTERESTS

K.H. and A.R.C. have intellectual property related to DNA nanoswitches. L.Z. and K.H. are inventors on a patent application covering aspects of this work. All other authors declare that they have no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Chemicals, peptides, and recombinant proteins</b>		
Agarose (molecular biology grade)	Sigma	Cat#A9539
Sodium dodecyl sulfate (20% Solution, RNase-free)	Invitrogen	Cat#AM9820
EvaGreen Plus Dye (20X in water)	Biotium	Cat#31000T
Poly(vinylsulfonic acid, sodium salt) solution	Sigma-Aldrich	Cat#278424
10X CutSmart buffer	New England Biolabs	Cat#B7204S
GelRed nucleic acid stain (10,000X in water)	Biotium	Cat#41003
Ficoll 400 (Biotechnology grade)	VWR	Cat#89399-840
Bromophenol Blue	Fisher Scientific	Cat#115-39-9
Polyethylene glycol (PEG)-8000	Thermo Fisher	Cat#BP233
<b>Critical commercial assays</b>		
TaqMan microRNA Assay (Assay ID: 000391)	Thermo Fisher	Cat#4427975
Luna universal one-step RT-qPCR kit	New England Biolabs	Cat#E3005S
TaqMan miRNA ABC Purification kit - Human panel A	Thermo Fisher	Cat#4473087
<b>Oligonucleotides</b>		
M13mp18 single-stranded DNA	New England Biolabs	Cat#N4040S
All other oligonucleotides	Integrated DNA Technologies (IDT)	
<b>Other</b>		
BtsCI enzyme	New England Biolabs	Cat#R0647S
DNAse I enzyme	New England Biolabs	Cat#M0303S
HiScribe T7 High Yield RNA Synthesis Kit	New England Biolabs	Cat# E2040S
HeLa cell total RNA	Biochain	Cat#R1255811-50
MCF-7 total RNA	Biochain	Cat#R1255830-50
Zymo Oligo Clean & Concentrator Kit	Zymo Research Corporation	Cat#D4060
Zymo Gel RNA recovery kit	Zymo Research Corporation	Cat#R1011

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ken Halvorsen ([khalvorsen@albany.edu](mailto:khalvorsen@albany.edu)).

#### Materials availability

This study did not generate new unique reagents.

#### Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

### METHOD DETAILS

#### DNA nanoswitches

All DNA nanoswitches were designed and fabricated using previously established protocols ([Chandrasekaran et al., 2019, 2020](#)). Briefly, circular M13 ssDNA (New England Biolabs) was linearized using the enzyme BtsCI (New England Biolabs). The linearized

M13 was mixed with 10× excess of backbone and detection oligos (Integrated DNA technologies, IDT) (sequences of all oligos are provided in [Table S2](#)) and annealed in a thermal cycler from 90 to 25°C at 1°C/min. After construction, DNA nanoswitches were purified by either HPLC or by PEG precipitation as described previously ([Chandrasekaran et al., 2020](#)).

### RNA samples

The 401 nt mRNA fragment was produced by *in vitro* transcription (NEB, HiScribe T7 High Yield RNA Synthesis Kit) from a DNA template. MicroRNA targets (miR-16 and miR-206) were purchased from Integrated DNA Technologies (IDT). Total RNA from HeLa cells and MCF-7 cells was purchased from BioChain Institute Inc. The RNA strand containing the m<sup>4</sup>.<sup>4</sup>C modification was synthesized in house at 1.0 mmol scale by solid phase synthesis using an Oligo-800 synthesizer. After synthesis, the oligos were cleaved from the solid support and fully deprotected with AMA (ammonium hydroxide:methylamine solution = 1:1) at 65°C for 45 min. The amines were removed by Speed-Vac concentrator followed by triethylamine trihydrofluoride (Et3N.3HF) treatment for 2.5 h at 65°C to remove the TBDMS protecting groups. RNA was then cooled down to room temperature and precipitated by adding 25 μl of 3 M sodium acetate and 1 ml of ethanol. The solution was kept at –80°C for 1 h before the RNA was recovered by centrifugation and finally dried under vacuum. The RNA strands were then purified by 15% denaturing polyacrylamide gel electrophoresis (PAGE) and were desalted, concentrated and lyophilized before redissolving in RNase free water.

### Nanoswitch detection assays

Nanoswitch detection assays were typically performed in 5–10 μl volumes with ~0.2–1 nM of nanoswitch in tris-HCl buffer supplemented with 30 mM magnesium and incubated at an elevated temperature. In general, we found 30°C to be most effective for microRNA targets and 40°C to be most effective for longer RNA targets. Additives such as SDS (0.05% final concentration) and short random blocking oligos (200 nM final concentration) were sometimes used to help prevent RNA sticking to the tubes, especially in cases where total RNA was not present.

Slight variations between experiments are further explained below. For miR-16 detection assays, we used a 5 μl reaction containing ~0.4 nM nanoswitch, 20 mM Tris-HCl, 30 mM MgCl<sub>2</sub>, 0.05% SDS and target RNA at varied concentrations incubated at 30°C for 2 hours. The 401 nt mRNA fragment was treated the same except with a 40°C incubation for 2 hours. For multiplexing experiments, we used ~0.8 nM of nanoswitch, 1× PBS with 10 mM MgCl<sub>2</sub>, and an overnight thermal annealing ramp (40 to 25°C, 0.1°C/min) to ensure both targets were captured. For non-synthetic RNAs (i.e. IVT or total RNA) we heat treated for 10 minutes at 75°C with 5 mM EDTA to ensure no residual DNase activity.

Gel stain and a Ficoll-based loading dye were added to samples before gel electrophoresis. 1× GelRed was used for all gel images shown in the paper; 2× EvaGreen was used for blue-light excision of samples that were quantified in [Figures 2](#) and [3](#). Gel electrophoresis was performed using 0.8% agarose gels in 0.5× TBE buffer prepared with nuclease free water. Gels were run at 60–75 V for 45 minutes to 2 hours depending on the assay.

### RNA purification protocol

After performing the nanoswitch detection assay and running gel electrophoresis, the looped nanoswitch gel bands were excised either on the UV transilluminator of a Bio-Rad Gel Doc XR+ System (for GelRed stained samples) or on an Invitrogen blue light transilluminator (for EvaGreen stained samples). A disposable plastic gel cutting razor (Sigma-Aldrich) was used to excise bands and to dice them into small pieces before being transferred into 1.5 ml tubes. Then, the gel pieces were weighed to estimate volume and brought up to a total volume of 100 μl or 200 μl containing 1× DNase I buffer (NEB) and 2 U DNase I (NEB) and incubated at 37°C for 1 hour. Following DNase I digestion, the RNA was recovered by either a modified “crush and soak” method (optimized for microRNA) or a modified Zymo Gel RNA recovery kit (optimized for longer RNA). Polyvinyl sulfonic acid (PVSA) (Sigma Aldrich) was also included (45 μg/ml final concentration) as an RNase inhibitor during final RNA purification, prior to quantification by RT-qPCR.

The crush and soak method proceeds by spinning down the gel pieces in a 0.2 μm filter (VWR) for 2 minutes at 12,000xg, and then re-submerging the gel pieces in ~100 μl of nuclease-free water for an additional 5 minutes at room temperature before performing a second spin. The collected material was then used as the input for an oligo clean up kit (Zymo Oligo Clean & Concentrator Kit). The manufacturer instructions were followed except for a prolonged 5 minute spin after the wash step to completely dry residual ethanol, and a 2× sequential elution using 50°C water and incubated 5 minutes at room temperature before each elution.

The gel recovery method proceeds by using the Zymo Gel RNA recovery kit with minor modifications as follows. Gel is denatured in twice the manufacturer’s recommended volume of proprietary RAD buffer at 55°C for 10 minutes, and again we used a prolonged 5 minute spin after the wash step to dry completely, and a 2× sequential elution using 50°C water and incubated 5 minutes at room temperature before each elution.

### Modification analysis by LC-MS

Measurement of the level of m<sup>4</sup>.<sup>4</sup>C was performed by ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) using a method similar to that previously described ([Basanta-Sanchez et al., 2016](#)). First, we digested the RNA with m<sup>4</sup>.<sup>4</sup>C modification using the Nucleoside Digestion Mix (NEB, M0649S) in 15 μl volume and used 10 μl for the test. The UHPLC-MS/MS analysis was accomplished on a Waters XEVO TQ-S<sup>TM</sup> (Waters Corporation, USA) triple quadruple tandem mass

spectrometer equipped with an electrospray source (ESI) maintained at 150°C and a capillary voltage of 1 kV. Nitrogen was used as the nebulizer gas which was maintained at 7 bars pressure, flow rate of 1000 l/h and at a temperature of 500°C. UHPLC-MS/MS analysis was performed in ESI positive-ion mode using multiple-reaction monitoring (MRM) from ion transitions previously determined for  $m^{4,4}C$  ( $m/z$  272 > 140). A Waters ACQUITY UPLC™ HSS T3 guard column (2.1 × 5 mm, 1.8 μm) attached to a HSS T3 column (2.1 × 50 mm, 1.7 μm) was used for the separation. Mobile phases included RNase-free water (18 MΩcm<sup>-1</sup>) containing 0.01% formic acid (Buffer A) and 50% acetonitrile (v/v) in Buffer A (Buffer B). The digested nucleotides were eluted at a flow rate of 0.4 ml/min with a gradient as follows: 0-2 min, 0-10% B; 2-3 min, 10-15% B; 3-4 min, 15-100% B; 4-4.5 min, 100 %B. The total run time was 7 min. The column oven temperature was kept at 35°C and sample injection volume was 10 μl. Three injections were performed for each sample. Data acquisition and analysis were performed with MassLynx V4.1 and TargetLynx. Calibration curves were plotted using linear regression with a weight factor of 1/concentration (1/x).

### RT-qPCR assays

Quantification of RNAs using RT-qPCR was carried out using the two-step TaqMan microRNA Assay (Thermo Fisher) for miR-16, and the Luna universal one-step RT-qPCR kit (New England Biolabs) for the rRNAs and mRNA fragment. Manufacturer's protocols were followed, and experiments were carried out using the MyGo Mini S real-time PCR instrument (Azura genomics) or a Bio-Rad CFX Thermal Cycler. Primers used in the one-step RT-qPCR are reported in the oligonucleotide list. To compare pre- and post-purification, the same amount of input RNA and final volume was used for both positive controls and for purified products so that C<sub>q</sub> values could provide a direct comparison. The  $\Delta\Delta C_T$  method was used for relative quantification of RNA yields. RT-qPCR experiments in [Figures 2](#) and [3](#) were performed using experimental duplicate or triplicate. Samples that did not amplify were excluded from analysis. Automated analysis of C<sub>q</sub> values was determined using the instrument's included analysis software.

### Beads purification

Purification of miR-16 using magnetic beads was performed using the TaqMan miRNA ABC Purification kit – human panel A (Thermo Fisher). Manufacturer's instructions were exactly followed.

### QUANTIFICATION AND STATISTICAL ANALYSIS

Gels were analyzed and band brightnesses quantified using Bio-Rad ImageLab software. qPCR data was analyzed by the manufacturer included software, either the MyGo Mini PCR software (Azura genomics) or CFX Manager (Bio-Rad). C<sub>q</sub> values were determined by auto quantification in each software, and relative quantification determined by the  $\Delta\Delta C_T$  method. Mass spec data was analyzed with MassLynx software, with amounts determined by automated peak integration. Relative quantification was based on use of a standard with calibration curves plotted using linear regression with a weight factor of 1/concentration (1/x). Details about replicates and error bars are described in figure legends.