cArgo: an Argonaute-Mediated Viral Point-of-Care Diagnostic Device

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Abstract

The COVID-19 pandemic strained global diagnostic capacities and highlighted the limitations of conventional lab-based assays. Early pandemic testing kits were reported to have false-negative rates as high as 29%. In an effort to provide accurate, non-invasive, affordable, and rapid Point-of-Care (POC) diagnostic tests for COVID-19 and other emerging pandemics, Purdue iGEM has spent the previous two years working on cArgo: an Argonaute mediated microfluidic diagnostic device which can be adapted to detect any viral pathogen. cArgo extracts viral RNA from saliva for amplification and conversion into dsDNA. The TtAgo Argonaute protein then cleaves the dsDNA using viral strain-specific DNA guides producing ssDNA fragments. These fragments serve as secondary guides allowing the Argonaute to cleave the molecular beacon emitting a quantifiable fluorescent signal for conclusive result determination. Coupling the biologics with chip barcoding and app integration, we hope to revolutionize POC Diagnostics while making data more accessible for simultaneous detection and contact tracing.

Introduction

Without a doubt, COVID-19 changed our world. As a result of the widespread physical, economical, and mental impact on both larger infrastructures and on individuals, over 5 million individuals have lost their lives to the ongoing coronavirus pandemic. The virus also stalled economic growth, most notably seen in supply chain issues that arose with an increased need for proper PPM, sterile materials, and testing kits. The pandemic highlighted issues in our reliance on global supply chain and plastic manufacturers to provide many of the materials used in diagnostic tests, such as nasal swabs and other pieces for rapid diagnostic kits. Even then, many kits that were able to be bought and used were unreliable with high false positive and false negative rates, and results could take up to 14 days in some instances to obtain. In April 2020, UC Davis conducted an analysis regarding the current false-negative rates of RT-PCR SARS-CoV-2 which concluded that early pandemic diagnostic assays had false-negative rates between 2% to 29%, indicating up to 29% of patients tested could have a false negative result [1].

Seeing the compromised detection accuracy in overall PCR and swab testing, we set out to create a rapid fluid-diagnostic kit that was able to provide accurate results. Noting the importance of proper testing protocols as an effective way to mitigate the spread of the deadly virus as well as other emergent viruses in the future, it was important the the device could be used point-of-care in an easy and adaptable manner such that it could be modified as other superbugs and pandemic-like viruses emerge.

The biologics of our device are based on previously implemented argonaute-mediated nucleic acid viral diagnostic systems. Recently developed diagnostic assays for COVID-19 have successfully implemented the argonaute protein of archeon Pyrococcus furiosus (PfAgo), inspiring us to follow suit [2,3]. We specifically decided to use argonaute technology over existing Cas13-based nucleic acid diagnostic assays as argonaute proteins maintain a lower star activity and do not require a PAM sequence for specific, directed cleavage. We specifically chose to use *Thermus thermophilus* argonaute (TtAgo) for our detection based on its reaction temperature between 65C-85C, much lower than that of other argonaute proteins.

The diagnostic assay to be implemented in our device cArgo included four main steps: RNA isolation via chitosan adsorption and elution, conversion of RNA into dsDNA before isothermally amplifying the target sequence with RPA, using TtAgo to cut our single-stranded target DNA sequence from amplified double-stranded DNA, and molecular beacons which will produce fluorescence upon recognition of the complementary target sequence. As the RPA primers, argonaute guide sequences, and molecular beacon probe sequence must be compatible with one another for the system to effectively detect a given sequence, we developed a biological part design program using Python. Our program generated optimal sets of RPA primers, tTago guide sequences, and molecular beacons given a viral genome. With this software tool, our diagnostic assay could be easily adapted to fight against the spread of new viral pathogens by providing researchers an efficient way to redesign pathogen-specific biological parts.

Following the development of a diagnostic assay, we investigated existing point-of-care testing methods in which our assay could reasonably be performed. We decided to implement a microfluidic chip due to the rapid nature of microfluidic systems. Our diagnostic is used as explained in the following sentences. First, the patient's saliva is inputted into the microfluidic chip and flows through an embedded chitosan capillary, extracting RNA from the saliva. After the RNA is eluted, the RNA continues into the reaction chamber where it is converted to DNA and amplified to produce dsDNA of our target sequence via RT-RPA. Argonaute proteins that have previously been incubated with DNA guide sequences and molecular beacons with probes complementary to the ssDNA target sequence then enter the

reaction chamber [2,3]. Here, argonautes cleave the amplified dsDNA to generate ssDNA. The produced ssDNA fragments then bind to molecular beacons, causing the fluorophore to and quencher of on the 3' and 5' ends of the beacon to separate as the hairpin structure hybridizes and is eventually also cleaved by the argonaute. A fluorescent signal is emitted and can be further analyzed to determine the viral load within the saliva.

Upon regaining wet lab access in 2021, we began to validate and characterize individual steps of our molecular assay. Chitosan RNA extraction, RPA, and argonaute cleavage all proved to be functional using the biological parts designed the previous year using our Python program. We were unable to validate the molecular beacon but plan to continue testing it to determine optimal experimental conditions. Therefore, we conclude that a majority of the parts of our molecular diagnostic assay are functional when used to target a characteristic sequence of SARS-CoV-2.

Results

Biological Part Design

Our written program successfully generated DNA sequences for all three essential biological parts of diagnostic assay: identification of a target, generation of appropriate guide DNA sequences for TtAgo, design of primers suitable for Recombinase Polymerase Amplification of previously identified target sequence, and design and classification of compatible molecular probes. We found use of .txt files saved computational power considerably from other existing software [4] and prevented having to run the same sequence multiple times. Our final code takes as an input a .txt file containing a whole genome sequence of an organism of interest (entered in 5' to 3' direction) and returns the most suitable target for a probe sequence, a .txt file containing RPA amplification targets sorted based on GC% of their gn, respective primers according to the Primer3 design parameters, and another .txt file with optimal beacons for the selected probe sequence sorted according to increasing ΔG.

Chitosan RNA Extraction

RNA Adsorption Capacity

To characterize and compare adsorption capacity of each material, we evaluated RNA adsorption of each material through isotherm model experiment and fitting them to a Langmuir isotherm adsorption model, previously utilized for characterizing similar nucleic acid adsorption systems [6].

Figure 1 shows chromatography has a much higher RNA capacity than glass fiber. Similarly, for both materials, the adsorption seems to be highly concentration dependent. Despite the RNA available being much higher than the total RNA bound, the amount of RNA bound only increases significantly when the starting concentration does; further supporting the equilibrium relationship nature of the adsorption process. The isotherm parameters for each material are summarized in Table 1.

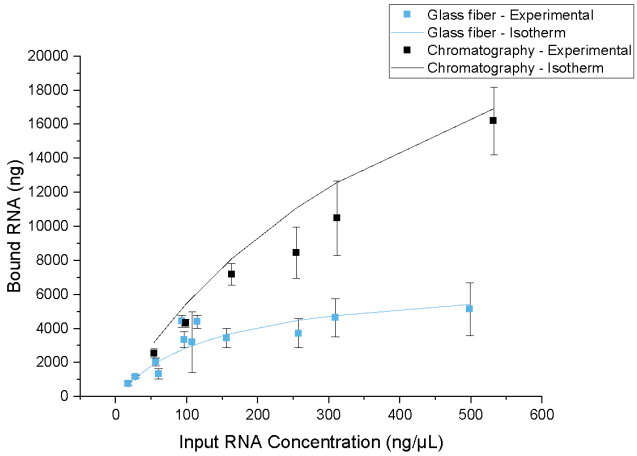

Figure 1. Experimental data and equilibrium curve derived from Langmuir isotherm model for both chromatography and glass fiber data.

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Table 1: Langmuir isotherm constants for Standard 17 glass fiber and Grade 1 chromatography RNA adsorption equilibrium curves.

Material	Q_{max}	K_{eq}	R^2_{adj}
Standard 17 Glass fiber	6966.235	0.007	0.91204
Grade 1 Chromatography	32806.499	0.002	0.99525

As exhibited from Q_{max}, the maximum RNA bound to each material is nearly 4.5-fold higher for chromatography than for glass fiber. However, the equilibrium constant K_{eq} for Grade 1 chromatography is lower, indicating that the curve is also bound to have a steeper descent as input concentration decreases. We presume this difference is due to the material's inherent properties, namely grade 11 chromatography is thicker and has larger pore size which increases surface area and thus number of available sites for RNA binding; as well as cellulose's natural affinity to nucleic acids due to electrostatic interactions [7].

Effect of Sample Volume

We further determined our system's versatility to a variety of sample and elution volumes, given the different reaction volumes and sample origins Point-Of-Care diagnostics might encounter [8]. We diluted the same amount of input DNA (~8500 ng) to various volumes of 50 mM MES and determined RNA eluted in 100 μL Tris buffer (Figure 2).

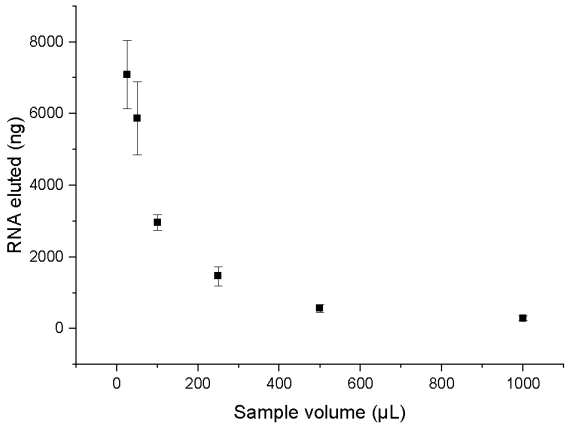

Figure 2. RNA eluted from Standard 17 glass fiber pads in 100 µL Tris buffer for a sample input of 8500 ng RNA diluted in different volumes of 50 mM MES.

From results seen in Figure 2, larger volumes, meaning more dilute samples, resulted in the poorest RNA recovery rates whereas more concentrated samples exhibited the highest recovery, with nearly all the RNA being recovered. This trend further supports data exhibited from the RNA adsorption isotherms which showed high concentration dependence of our adsorption platform and suggest overall recovery will be limited by initial concentration rather than amount of RNA in the sample.

Effect of Elution Volume

We determined the optimum volume range under which to elute the nucleic acids from our platform to obtain the maximum amount of RNA without unnecessarily diluting the sample. For a fixed sample of 100 µL sample of 80 ng/µL yeast RNA in 50 mM MES. We determined the concentration and amount of RNA eluted for each different elution volume (Figure 3).

Figure 3B exhibits a similar equilibrium curve as the one seen in the RNA adsorption isotherms. Given the equilibrium nature of the adsorption reaction, larger elution volumes result in a larger amount of RNA being released from the pad to reach equilibrium. However, Figure 3A shows for volumes larger than 50 µL the concentration of the eluant only decreases. While larger elution volumes correlate to higher RNA yields, at volumes larger than 100 µL this gain becomes less noticeable. Furthermore, typically nucleic acid reaction volumes are unlikely to exceed 100 µL without becoming prohibitively expensive. Therefore, we estimate elution volumes between 50-100 µL to be a suitable compromise between high RNA yields and sufficiently small reactions.

Recombinase Polymerase Amplification (RPA)

Due to the safety concerns, we were unable to work with COVID-19 RNA. Thus, we decided to evaluate our primer design and test the efficacy of TwistDx RPA amplification using DNA. Using the primers designed by our Python program, we ran RPA using the target double-stranded DNA as our template. Testing the primer was successful as it showed amplification of the short (0.142 kilobase pairs) target SARS-CoV-2 sequence (Figure 4). The amplified product was used for cleavage assay using the argonaute protein in downstream assays.

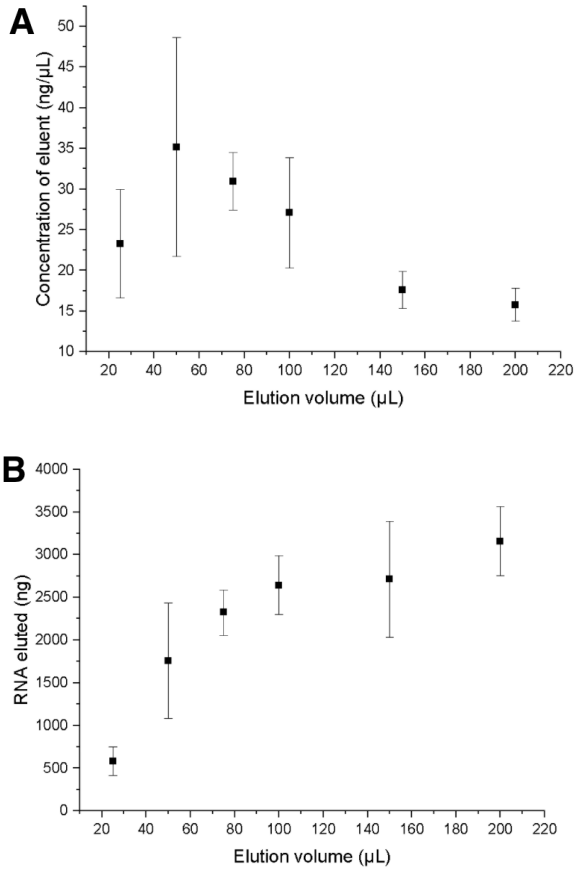

Figure 3. Concentration (A) and amount (B) of RNA from different elution volumes of a Standard 17 glass fiber pad treated and a fixed 100 uL sample of 80 ng/uL.

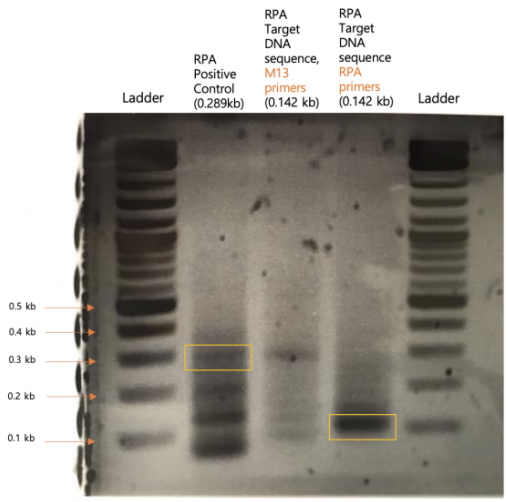

Figure 4. Agarose gel (1%) with RPA-amplified sequences. The fourth lane includes RPA-amplified SARS_CoV_2 sequence with primers designed using the biological part design Python program.

Protein Expression of H2Ago and TtAgo

The biologics of cArgo chip is based on argonaute protein, which we decided to clone and produce at our capability. We worked to express and purify the Argonaute protein Thermus Thermophilus or TtAgo in E. coli. Along with the expression and purification of TtAgo in E.coli, we tried to express and purify a previously cataloged iGEM part, another Argonaute protein called h2Ago. Upon troubleshooting we were able to successfully clone the codon-optimized sequences of both argonaute proteins into DH5 alpha and BL21 strains of *E. coli* to further continue the protein expression process. The results showed non-ideal purification and insufficient lysis of the cells. Overall, the approach to quantify expression via purification was non-ideal. Other methods of characterization should be investigated instead.

TtAgo Cleavage

The argonaute protein in the cArgo chip cleaves the double stranded DNA product from RPA and uses the product to guide a secondary round of cleavage on the molecular beacon. To produce the single-stranded DNA guide from the target double-stranded DNA, the three phosphorylated guides designed from the biological part Python program were used. When run on a Urea-PAGE gel, the desired band representing the single stranded DNA sequence was present (Figure 5).

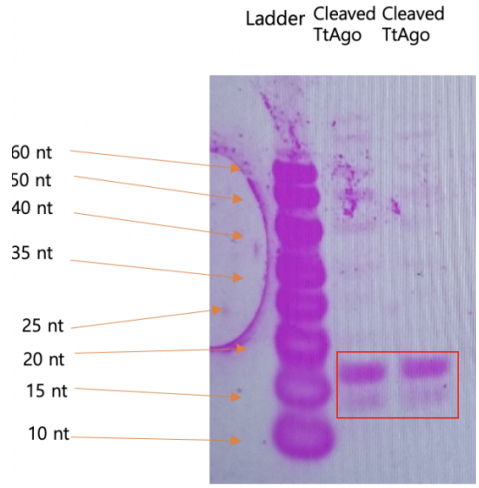

Figure 5. Urea-PAGE gel to capture the target DNA sequence. TtAgo cleavage assay was performed with 1 uL of each guide DNAs. The expected bands to be seen include 66, 16, and 25 nt.

Molecular Beacon Fluorescence

The device was designed to measure a quantitative fluorescent signal for conclusive result determination. To test the secondary cleavage and final fluorescence quantification, a combined cleavage and fluorescent assay was conducted. A reaction of 5pmol of the beacon, 10pmol of TtAgo, 3pmol of each DNA guide, and 1pmol of the target dsDNA at a reaction volume of 35ul with reaction buffer (which contained 20 mM HEPES pH7.5, 250mM NaCl, and 0.5 mM MnCl2) was incubated at 80C for 30 min on a dry bath and then cooled to room temperature. The samples were pipetted into a well plate and a fluorescent and absorbance reading was taken.

Conclusions

Throughout our series of experiments, we have successfully validated the functionality of chitosan RNA extraction, amplification through RPA, and TtAgo cleavage using biological parts designed to identify the presence of a sequence from SARS-CoV-2.

Our experimentation on chitosan proved its capacity as an extremely simple, instrument free platform for nucleic acid capture which takes only a few minutes to carry out. The chitosan-treated materials tested maintained versatility to a variety of sample and elution volume and determined due to high concentration dependence the system works better under low sample volumes when amount of RNA is fixed. However, this same concentration dependency suggests that for dilute samples, large sample volumes might return higher yields as higher amounts of RNA will be recovered from the sample at higher volumes if the concentration is maintained constant. Finally, regarding elution volumes we demonstrated our platforms behaves best within the range of typical reaction volumes for nucleic acid reaction platforms of 50-100 µL.

Reverse polymerase amplification (RPA) was proven to be effective when used to generate a high concentration of the target sequence from SARS-CoV-2. When run on a 1% agarose gel, a dark band around 142 bp, the length of our target, is shown, indicating reaction success. Thus, the RPA reaction itself and the primers designed from our Python program are functional.

While we were unable to perform a cleavage assay on codon-optimized TtAgo expressed from *E. coli*, we observed successful argonaute cleavage when 3 uL of each of our three phosphorylated guide sequences are used with NEB tTago. The functionality of TtAgo also suggests our Python program is capable of designing a set of compatible biological parts.

We did not obtain conclusive results from experiments performed on our molecular beacon. More experimentation will be required to confidently determine whether or not the molecular beacon suggested by our Python program works.

With exception of the molecular beacon, the proposed molecular diagnostic assay and the biological parts designed using our developed Python program proved to be successful when used with a characteristic sequence of SARS-CoV-2. Thus, we conclude that the diagnostic assay of cArgo is mostly functional and has potential to be applied to diagnose viral infections other than COVID-19 with the use of our Python design program.

Biosafety Concerns

As the diagnostic assay of cArgo does not use volatile chemicals, the dominant biosafety concern related to our device is exposure to potentially biological samples. If a saliva sample is present on or leaks from the device after use, this could pose a potential biohazard to the user or the user's surroundings. Therefore, used devices should ideally be collected, handled, and disposed of by trained medical professionals.

The buffer solutions used to mediate reactions within the device may pose a hazard if touched with bare skin or ingested, so caution should be taken in the manufacture of the device to ensure no leaks occur.

Materials and Methods

Biological Part Design Program Development

Molecular Beacon Design:
In order to easily detect when our RNA fragment of interest is present, it is necessary to use some form of probe which will emit a visible signal with high specificity to the molecule of interest. For our project, a molecular probe specific to the TtAgo guide DNA was selected for coupling our detection system. However, currently available software for design of molecular beacons was often expensive and inaccessible, hence we decided to develop our own molecular beacon design software. Literature [5] suggested G-C content of the stem to be between 70-80% and avoiding sequences with a 5' guanine. The stem length of the beacon should be in between 4-7 nucleotides.

Our initial approach took a specific probe sequence from the COVID genome. It was then screened for all possible stems according to the guidelines outlined above. The probe and stem combine sequence was uploaded to Quikfold to determine sequences which folded into the correct hairpin structures. From the sequences that did fold into hairpin structures, they were manually screened to see any inconsistencies with the outlined parameter.

Target Sequence & TtAgo Guide DNA Design:
Initially, we searched current literature and used the Basic Local Alignment Search Tool (BLAST) to identify unique sequences specific to Sars-CoV-2. However, further automation was pursued to identify a specific target compatible for Argonaut detection assays.

We designed guide nucleotides (gt, gn, gr, gf) by entering a whole genome sequence and scanning for a section of the genome which met the following characteristics as outlined in literature [9,10] (for all positions, genome is being read 5' to 3'):

For gn:
- First position starts with T
- 12th position is A

For gf:
- Position 10 nt upstream of 5' start of gn is a T
- 12th position is G or C

For gt:
- From 3' end of gn, 10 nt upstream is a T
- 12th position is G or C

For gr:
- Base pair 10 nt upstream of 3' end of gn is a T
- 12th position is G or C
- GC content between 12-38%

Primer Design
We designed RPA primers following recommendations from TwistDx and utilizing the open-source software Primer3 under the following conditions:

Primer length: 30-36 bp
GC content: 30-70%
Melting temperature: 50-100 °C
Product range: 100-200 bp
Maximum repeat: 5 bp
Initially, we searched current literature and used Basic Local Alignment Search Tool (BLAST) to identify unique sequences specific to Sars-CoV-2. However, further automation was pursued to identify a specific target compatible for Argonaut detection assays.

Scaling up:
Given our design approach required a considerable amount of tedious data entry into various websites by the user, we implemented web scraping in Python by using the Selenium library to construct a more robust and scalable code. Through this library, we were able to automate interaction of Primer3 and Quikfold to minimize user intervention.

Chitosan RNA Extraction

Reagent and material preparation

Low molecular weight chitosan with 75-85% degree of deacetylation was purchased from Sigma Aldrich. Yeast total RNA 5 mg/mL was purchased from ThermoFisher Scientific. Standard 17 glass fiber pads were purchased from Cytiva. (3-Glycidyloxypropyl) trimethoxy silane (GTPMS), Whatman Grade 1 chromatography paper and Tris buffer were purchased from Fisher Scientific.

Solutions of 50 mM MES and 50 mM Tris buffer were prepared in sterile, reverse osmosis purified water and pH was adjusted to 5.0 and 9.0 respectively.

Chitosan pads preparation

A solution of 0.1% v/v GTPMS with 1% w/v chitosan in 0.1M acetic acid was prepared. Pads of 5x5 mm of either Standard 17 glass fiber or Grade 1 chromatography paper; were cut using a custom mechanical hole puncher. Pads were left soaking in the chitosan solution overnight. Pads were then blotted on a Kimwipe (Kimberly Sciences), rinsed with 50 mM MES buffer, blotted on a Kimwipe again and left on a clean Petri dish overnight to dry.

RNA extraction protocol

Yeast RNA was diluted in 50 mM MES buffer. Pads were placed individually on 1.5 mL microcentrifuge tubes. Afterwards, 100µL RNA solution was pipetted into the tube. Each pad was then transferred to a new tube containing 100µL Tris buffer. Concentration of starting RNA solution, binding solution and elution buffer were measured with a Nanophotometer (IMPLEN N60).

Data analysis was conducted using Origin 2020b.

Protein Expression of TtAgo and H2Ago

Codon optimization was done on both TtAgo and h2Ago to attempt to improve protein expression efficiency. Both constructs were cloned into the backbone from plasmid pWUR702 [x]. Several difficulties occurred during the cloning process. WideSeq sequencing revealed the incorrect transformation of both constructs. In order to troubleshoot seeing no transformants, we attempted to replace the codon-optimized argonaute insert with noncodon optimized TtAgo, which was proven to be a successful ligation to validate cloning protocols and procedures taken. Additionally fresh reagents and high-fidelity double digest restriction enzymes were ordered.

Expression of TtAgo and H2Ago was attempted using the codon optimized TtAgo and H2Ago cloned within the E. coli BL21 strain. BL21 (DE3) strain is commonly used in practice for protein expression as T7 RNA polymerase expression as well as protease production is inhibited. Expression of argonaute proteins was based on slow induction [11]. Induced cells were then lysed by sonication and purified using ultracentrifugation as well as protein column purification. We used His-tag protein purification columns with protease inhibitors to minimize protein degradation. The purified protein as well as lysates were visualized on a 8% SDS-PAGE gel.

Recombinase Polymerase Amplification

Recombinase Polymerase Amplification is the amplification technique we have chosen to employ due to several advantages. It is portable and isothermal so expensive lab equipment such as a thermal cycler does not have to be employed. The amplification time takes less than 30 minutes at 37°C. To amplify our target double-stranded DNA, we added the reagents included in the TwistAmp® Liquid Basic kit (TwistDx), primer, and template and incubated the reaction for 30 minutes at 37°C. We then ran our products on a 1% and 2% agarose gel to evaluate if the reaction was successful. One sample with the target COVID sequence along with one sample with h2ago was run as control. Another sample was run with the positive control provided within the TwistDx RPA kit. The bands present on the agarose gel for the negative and positive control were used to qualitatively gauge if our reaction is successful.

TtAgo Cleavage

To test functionality of our three phosphorylated DNA guides, we ran reactions at 80 C for 45 minutes using TtAgo purchased from NEB. 3 uL of each of the three guides was added to a 20 uL mixture containing 1 pmol of target DNA, 2 uL of 10X buffer, 1 uL of TtAgo, and nuclease-free water. Reaction success was evaluated by running products on a urea-PAGE gel to see if the ssDNA sequence is present.

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