

# The Development of a Highly Sensitive Home Diagnosis Kit for Group A *Streptococcus* Bacteria (GAS)

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## Summary

**Strep throat is a bacterial infection contracted by millions of people every year. Due to its similarity in symptoms to viral infections, a rapid diagnostic test to distinguish Group A *Streptococcus* bacteria (Strep A) from a viral infection is necessary for proper treatment. Any delay in treatment may cause secondary infections in the patients' sinus and/or kidneys. Current Strep A diagnosis methods, including bacteria culture and the rapid antigen detection test (RADT), are not as sensitive as molecular detection. Molecular detection is the ultimate tool for bacterial DNA detection, but often needs to be performed in a hospital laboratory. In this study, molecular technologies were incorporated into a home-based DNA detection assay to replace current bacterial detection methods. The molecular assay consists of three parts: DNA extraction on Flinders Technology Associates (FTA) filter paper for Micro Extraction Technology (MET), isothermal DNA amplification using Recombinase Polymerase Amplification (RPA), and bacterial detection through a lateral flow strip. We hypothesized that the rapid molecular assay possesses higher sensitivity in comparison to current detection methods. Controlled initial experiments showed that the molecular assay is 100-fold more sensitive than RADT, and has a low detection limit. Negative results using similar microorganisms, and the absence of cross reactivity support the specificity of the assay. Lastly, identical inter-run results suggest the assay has good reproducibility. Overall, this initial experiment provides potential for home molecular testing on Strep A patients. Further studies on the use of this assay on patients with Strep A infections will require clinical trials.**

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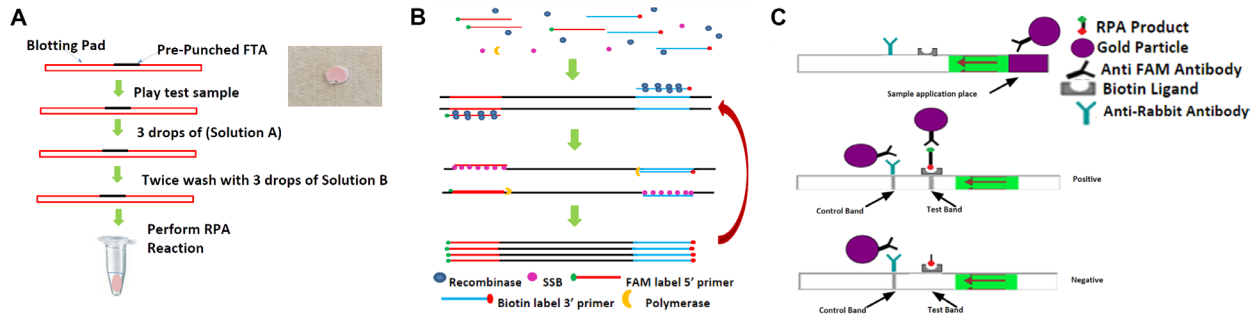
## Introduction

Strep throat is characterized by the inflammation of the pharyngeal region of the throat, which is infected by bacteria called *Streptococcus pyogenes*, also known as Group A *Streptococcal* bacteria (GAS). GAS can cause

many diseases, most commonly Strep throat, which induces immune system reactions such as fever, sore throat (pharyngitis), swollen lymph nodes, and tonsillitis. The majority of sore throat cases is caused by viral infections, while only about 30% of cases are caused by bacterial Strep throat infections (1). Whereas viral infections commonly resolve on their own, the infection with GAS must be treated with antibiotics. Without treatment, it may lead to secondary complications such as sinus, ear, heart (rheumatic fever), or kidney (post-streptococcal glomerulonephritis) infections (2). Since both the viral and bacterial diseases produce similar symptoms, a fast, differential diagnosis is necessary to determine whether the infection is caused by bacteria or a virus. Furthermore, disease-blind antibiotic treatment is not recommended, as it may contribute to the development of antibiotic resistance. The ability to differentiate between viral and bacterial infections could limit antibiotic output, potentially curbing the rapid development of antibiotic resistance.

Currently, there is a rapid antigen detection test (RADT) and a throat culture detection method, which is a gold standard for diagnosing strep throat. RADT is a fast diagnosis method that takes a few minutes and is based on antibody recognition of an antigen (3, 4). However, it only has a 60-70% sensitivity compared to the gold standard, cell culture. Bacterial cell culture is a traditional blood agar culture method. It has about a 90-95% sensitivity rate, and it also can detect the presence of less-common bacterial causes of pharyngitis (5). However, it takes 24 to 72 hours to get test results, often delaying treatment by late report. However, the molecular real-time PCR method not only achieves high sensitivity but also gets fast results within several hours (4). Therefore, detection of bacterial DNA molecules is a good way to achieve high sensitivity and specificity. However, in order to perform this Strep A bacterium molecular assay, tests must be performed in a hospital laboratory using a real-time PCR instrument, and they require a physician order, costing time and often delaying treatment. Allowing patients direct access to a highly sensitive test to perform at home in a point-of-care setting could overcome this barrier. Recent progresses in molecular diagnosis provide all the possibilities for developing a home-based molecular Strep throat test.

Traditional DNA extraction techniques require extraction instruments that are not feasible for point-of-care testing (POCT) or home molecular diagnosis kit development. In order to create a home diagnosis, DNA-based assay, the extraction technology used must be simplified. The Flinders Technology Associates (FTA)



**Figure 1. Schematics for (A) Micro Extraction Technology (MET) process using FTA filter paper, (B) the Recombinase Polymerase Amplification (RPA) Cycle and (C) the lateral flow detection system.** (A) Shows a blotting pad that contains a pre-punched FTA shown to the right of the diagram. Following steps include placing the swabbed sample, Solution A, and Solution B on the FTA and putting the FTA card in a tube for the RPA reaction. (B) Explains the RPA process involving an enzyme complex and primers. The recombinase leads the labeled primers to the target region, the single strand binding protein (SSB) stabilizes the structure, and the polymerase extends the strand. (C) The lateral flow test implements gold particles that correspond with the labeled ends of the amplified DNA to form a test band and a control band. A positive reaction contains two dark bands and a negative reaction only contains one dark band.

card fits these requirements (6). The surface of the FTA card includes proprietary chemicals that assist in cell lysis through direct contact, trapping nucleic acids with positive electrostatic attractions within the fibers. The remaining protein and lipids are removed with the wash solution applied and the contacted DNA is purified and ready to use for molecular detection (**Figure 1A**). Isothermal DNA polymerase is the key technology used to perform non-instrument based nucleic acid amplification. Recombinase Polymerase Amplification (RPA) allows for targeted DNA amplification in a short period of time (7). It differs from traditional Polymerase Chain Reaction (PCR) techniques because RPA uses an isothermal cycle, making it possible to perform without the use of a thermocycler. The isothermal character of the RPA reaction is coupled with the use of the Recombinase enzyme, Single Strand Binding (SSB) protein, and DNA polymerase. First, Recombinase binds to fluorescent dye-labeled oligonucleotide primers. The complex (fluorescent dye, oligonucleotides, and recombinase) is matched and hybridized to the target region on the DNA strand and breaks the double strand to form a D-loop. The SSB protein attaches to the displaced DNA single strand to stabilize the D-loop, and the polymerase extends the strand based on complementarity to perform rapid DNA amplification under isothermal conditions (**Figure 1B**). After each strand template is duplicated and produces two templates, these will be duplicated again by the enzyme complex, rapidly amplifying the target templates *in vitro*.

The final part of the molecular diagnostic assay is the detection method, Lateral Flow. This is a traditional immunoassay for point of care testing using an antigen and antibody reaction (8). A DNA-based assay can also be used for lateral flow detection if the product is labeled with fluorescent dyes and biotin. The gold particles within the loading region are conjugated with the anti-FAM fluorescent dye antibody. Therefore, any analyte with a fluorescent label will bind to the gold particles. When the positive analyte flows from the loading point to the end of the strip through liquid capillary force, gold

particles will bind to the fluorescently-labeled analyte and move forward together. The movement stops when gold particles and analyte complex meet streptavidin, a protein that has a high affinity for biotin, and the gold particles accumulate to produce a visible, dark band in the positive detection region. The negative detection region contains anti-rabbit antibodies which bind to any conjugated gold particles (**Figure 1C**). Any gold particles without a positive analyte can continue to move toward the negative detection region, where they bind to any antibody and produce another dark band due to the gold particle accumulation in the negative detection region.

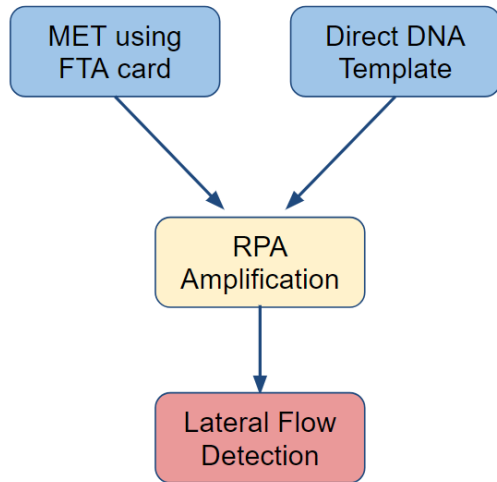
These novel molecular tools make a home-based assay for Strep A molecular testing feasible. Our goal was to apply these tools in order to create a sensitive and specific Strep A DNA-level test in the form of a home kit to provide patients with rapid access to test results before clinical confirmation. We hypothesize that a rapid molecular assay will possess a higher sensitivity, given advanced modern methods, in comparison to current rapid detection methods such as RADT for Strep A testing.

## Results

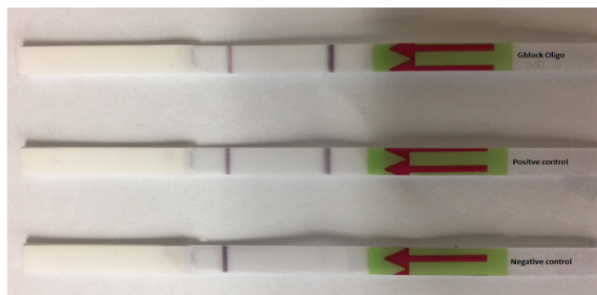
Using the combination of methodologies described above, we designed an RPA system appropriate for detecting Strep A (**Figure 2**). The first step to specifically detect Strep A is target selection. *Spy1258* is a putative transcriptional regulator gene, which is specific for Strep A and may be involved in species-specific maintenance or adaptation. We noted that gene sequences identical to *spy1258* were completely absent from all other bacterial genomes available at GenBank. Many studies have used this particular gene for the rapid detection of Strep A from various clinical samples with superior sensitivity and specificity (9). Therefore, we chose the GAS *spy1258* region to design the primers and used the double-strand gblock oligo DNA as the test template.

To assess the assay, Strep control DNA was tested, resulting in two dark bands, while the human DNA

negative control resulted in a single band, indicating that the test was working but Strep A DNA was not present (**Figure 3**). RPA can detect less than 10 copies of the template (7). Results from the gblock oligonucleotide template representing the GAS *spy1258* region test matched the two-band signal from the positive Strep A control (Figure 3), demonstrating that the selected GAS *spy1258* target DNA region was successfully amplified by RPA, and that the FAM and biotin dual-labeled DNA amplification product was successfully detected by the lateral flow detection system.



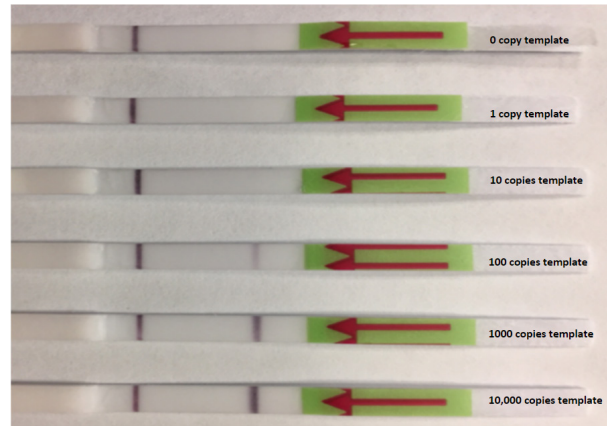
**Figure 2. Diagram of work process for the Strep A home detection kit.** This consists of three steps: DNA extraction, amplification, and detection.



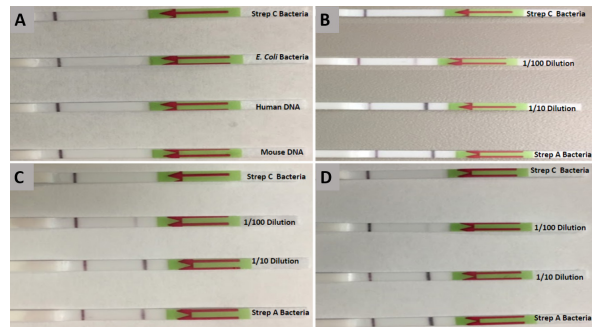
**Figure 3. Home detection kit assay results for the *spy1258* gblock oligo, Strep A bacterial positive control DNA, and human negative control DNA, from top to bottom.** Both the gblock oligo and the positive control read positive test results for Strep A (two dark bands). The negative control reads a negative test result (one dark band). Therefore, the limit of detection of this molecular assay was 100 copies of bacterial DNA. This result indicated that the molecular home diagnosis kit sensitivity is comparable to that of the PCR-based assay.

Next, we tested the sensitivity of the molecular assay using a gblock oligonucleotide series of dilution experiment. The successful detection of gblock oligonucleotides at 10,000 copies was verified from the experiment above.

We included concentrations of 10,000, 1000, 100, 10, 1, and 0 copies/ $\mu$ l to expand the dilution further. The 10,000, 1000, and 100 copy samples produced positive detection signals (two bands), and the 10, 1, and 0 copy samples produced negative detection signals (one band) (**Figure 4**). This result is specific to an analytic limit of detection (LOD), which was based upon a pure DNA template instead of a bacterial sample. There may be a difference between the analytic LOD and the true bacterial LOD.



**Figure 4. Home detection kit assay results for a dilution series of the gblock oligo, ranging from 0 copy templates to 10,000 templates.** 0, 1, and 10 copies template read negative results for Strep A (one dark band). 100, 1000, and 10,000 copies template read positive results (two dark bands). This indicates the limit of detection (LOD) for gblock oligo at 100 copies template.

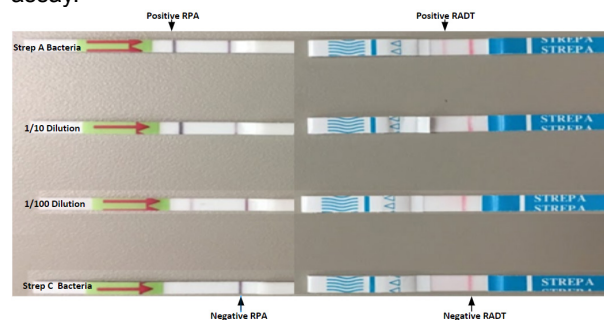


**Figure 5. Home detection kit assay results for four indicated samples to test for specificity (5A), with three additional repetitions to assess reproducibility (5B-D).** (5A) Test results indicate a negative detection for Strep A based on the presence of a single dark band. (5B-D) Test results show negative detections with the Strep C Bacteria and positive detections with the 1/100 dilution, 1/10 dilution, and the Strep A Bacteria sample.

We tested the specificity of the home-based assay using multiple samples from Strep C bacteria, E. coli bacteria, human genomic DNA, and mouse genomic DNA. All four samples produced negative results, indicating the specificity of the assay (**Figure 5A**). The assay reproducibility was tested using the positive Strep A

bacterial sample at 1/10 and 1/100 dilutions, as well as the negative Strep C bacterial sample. This experiment was run three individual times, and each experiment yielded the same results, indicating 100% reproducibility (Figure 5B-D).

To effectively compare the sensitivities of the two methodologies, RADT and the DNA home diagnosis, the home diagnosis method was tested using non-viable Strep A bacteria to mimic a real diagnosis environment. Moreover, the detection results of the DNA home diagnostic method were compared to the results from the RADT assay. The positive bacteria samples that were taken from the RADT kit were non-viable Strep A bacteria. The negative samples were non-viable Strep C bacteria. The Strep A bacteria were diluted in distilled water for a 1/10 dilution and a 1/100 dilution. The molecular assay showed a positive detection for Strep A at 1, 1/10, and 1/100 dilutions (Figure 6). The RADT assay only detected the undiluted positive sample and did not detect the 1/10 or 1/100 dilutions (Figure 6). Both tests yielded negative results for the negative control sample, indicating overall that the home-based assay was 100 times more sensitive compared to the RADT assay.



**Figure 6. Comparison of results from the home DNA diagnosis kit (left) with the rapid antigen detection kit (RADT) (right).** The home DNA diagnosis kit read positive test results for the Strep A Bacteria, 1/10 dilution, and the 1/100 dilution based on the two dark bands. RADT detected a positive result for the Strep A Bacteria, but it detected negative results for the 1/10 and 1/100 dilutions. Both assays detected negative results for the Strep C Bacteria (one dark band).

## Discussion

The development of a working home-based molecular assay is an important step in the diagnosis of Strep throat. The method implemented in this study significantly increased the accessibility to diagnostic information through the use of RPA (a fast, isothermal DNA amplification protocol) and a lateral flow strip. Through primary system testing and reproducibility testing, we confirmed that the home-diagnosis DNA assay can consistently identify Strep A infection. The limit of detection test showed the assay had a high sensitivity because it could detect the bacteria at low concentrations. This factor could impact the early treatment of disease and prevent the infection before it causes any severe symptoms. Finally, the test showed good specificity through successful DNA amplification of only the target Strep A antigen, without cross reaction

against other bacterial, human, or mouse DNA. This further demonstrated that the DNA-based assay is target-sequence specific, which increases the likelihood for the detection method to be applied to different infectious disease environments. However, it should be noted that the current testing was performed under ideal detection conditions, which may not correlate to a true diagnosis environment. In order to validate this diagnosis method, human clinical trials are necessary.

A home-based molecular DNA diagnosis kit was developed for Strep A detection by implementing MET, RPA, and lateral flow detection in sequence. This is the first time a DNA molecular test for Strep A diagnosis was performed successfully without laboratory support. The assay has an LOD at 100 copy using the bacterial sample and it is 100 times more sensitive than the antigen-antibody-based assay. The development of this home molecular DNA diagnostic assay shows promise for the future of POCT at home.

## Methods

### Materials

Sodium hydroxide pellets were purchased from Sigma-Aldrich, Catalog No. S588. Tris-EDTA Buffer was from Thermo Fisher Scientific (Catalog No.12090015). Hole punch and cotton swabs were from Walmart. Flinders Technology Associates (FTA) filter card was from GE healthcare (GE Healthcare, Catalog No. WB120305). Antibody Strep A detection kit was from NDC Inc. (Catalog No. P080033) and it included a 1 ml sample of non-viable Strep A bacteria as a positive control and a 1 ml sample of non-viable Strep C bacteria as a negative control. Recombinase Polymerase Amplification (RPA) enzymes mix was from TwistDx. Lateral flow detection strips were from Milenia Biotec universal strips MGH-1.

Primers were ordered from IDT DNA technologies (Coralville, Iowa). Primer sequences were selected from NCBI called serotype M1 *S. pyogenes* strain SF370 (GenBank accession number AE006565) targeted to *spy1258*. A portion of *spy1258* that covered the primer region was synthesized from IDT DNA technologies as gblock double strand oligonucleotides, which were used as positive control template. Sequences were as follows:

#### 1. Positive gblock oligonucleotide

```
ATGACCAAAAGACATACTGAAACCAAGCTTAT-
GTAAAGACCGCCTTAACCACCTTACTAAGTAA-
CAATCTTTTGAACTCTTACTGTCTCTGACCT-
TACTAAAAAGGCTGGTATCAATAGAGGAACCTTC-
TACCTCCACTACACTGATAAATTTGACATGATGAAT-
CACTTCAAAAATGACACTCTGGATGATTTGTACC-
GTTTGTAAATCAGGCTGAAATCTACACAGACACTC-
GACAAGTCCTCAATCAAACCTTGTCCTACCT-
TATAGAACATAGAGAATTTATAACCGCACTCGC-
TACTATTTCTTACCTCAAATTTCCGCAACTCAT-
CAAGGATTTCTGTTACCAATTTTGACTACTAT-
TACTGGTTTCCAAGACATTGTGACCAATCAATAC-
CATATTCCTTATCCCTATGCTTTAGAAGTTTACCTT-
GCCAGTATTGAGAGCATTATCAGC
```

2. *Forward dye-labeled primer*  
5'-/56FAM/TTGTACCGTTTGTAAATCAGGCTG  
AAATCTA-3'

3. *Reverse dye-labeled primer*  
5'-/5Biosg/TAGTAGCGAGTGCGTTATAAATTCTC  
TATGT-3'

Home Strep A diagnosis kit solutions were as follows:  
Solution A: 100 mM NaOH; Solution B: 10 mM Tris 0.1  
mM EDTA; Solution C: 10 mM Mg(CH<sub>3</sub>COO)<sub>2</sub>; Solution  
D: 0.5 μM forward and reverse primers; Solution E:  
Phosphate-Buffered Saline.

All reagent preparations were done in the hematology  
laboratory of the Mayo Clinic (Rochester, MN).

#### DNA extraction by MET

A cotton swab was used to collect Strep throat bacteria  
from a non-viable control sample. The cotton swab with  
a bacterial sample was transferred to a 5 mm diameter  
FTA card, which was prepared using a hole punch. The  
cotton swab was pressed on the FTA filter disk for five  
seconds for full contact with the FTA card. Three drops  
of Solution A (about 60 μl) were applied to the FTA  
disk for one minute. Then, the FTA disk was washed  
twice with three drops of solution B. After five minutes,  
the FTA Disk containing Strep A bacterial DNA was  
transferred to the reaction tube.

#### RPA Reaction Protocol

1 μl of diluted gblock oligonucleotide template (10,000  
copies/ul), 1 μl of Strep positive control, and 1 μl of  
100 ng/μl human DNA negative control were used. In  
the reaction tube, a lyophilized enzyme mix including  
recombinase, SSB, DNA polymerase etc. (TwistDx)  
was rehydrated by adding two drops of Solution C (~30  
μl). Then, two drops of Solution D (~30 μl) were added  
to begin the reaction. The reaction tube was heated  
by hand at around body temperature (~37°C) for 20  
minutes. Next, 20 drops of Solution E were added to  
the reaction mix to dilute the RPA reaction. The diluted  
reaction solution was then ready for detection by using  
lateral flow technology.

#### Lateral Flow Detection Protocol

One drop of the diluted RPA reaction sample was  
added to the sample loading region of the lateral flow  
strip. The strip was placed in a vertical position for  
three minutes and the bands identifying the positive  
or negative results appeared. A positive result was  
signified with two visible bands and a negative result  
with one visible band.

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