Title: Gold nanoparticle litmus assay for rapid diagnosis of Chlamydia trachomatis
Keywords: Chlamydia trachomatis, rapid diagnostic tests, amplification tests, immunotherapy, nanoparticles, optical
Specific Aim: In order to increase the specificity of recombinase polymerase amplification assays for detecting Chlamydia trachomatis, a seamless screening technique should be incorporated to detect false positives and false negatives. **I propose a method to identify false positive and negative results by conjugating gold nanoparticles to forward and reverse primers**. The presence of the Chlamydia target sequence leads to the aggregation of gold nanoparticles which creates a visual change in color. This secondary screening technique is incorporated during recombinase polymerase amplification, thus not affecting the overall turnaround time of the test.
Hypothesis: Gold nanoparticle conjugated primers can be used to improve the specificity of recombinase polymerase amplification-dependent Chlamydia tests without affecting the turnaround period. This method will provide a conspicuous visual color change which detects false positives and negatives.
Background: Diagnostic tests for viral sexually transmitted diseases (STDs) provide specific and rapid turnarounds with the use of an enzyme-linked immunosorbent assay (ELISA). HIV antibody detection with DPP® HIV-Syphilis Assay presents a turnaround time of 10 minutes and 98.7% specificity [1]. However with bacterial STDs, the challenge of reducing the turnaround time while maintaining specificity still exists. The most specific and standard diagnostic test for *Chlamydia trachomatis* (CT) is Nucleic Acid Amplification Tests (NAATs) which rely on PCR amplification and extraction of nucleic acids. Although NAATs yield the most accurate results, there is a longer turnaround time of several hours [2-3].

Rapid diagnostic tests in CT detection has reached a turnaround time of 30 minutes with a single chip device known as The Velox™ technology that is comprised of software and electronics to extract DNA, amplify via polymerase chain reaction, and detect CT sequences with electrochemically-labeled DNA probes [4]. However, the one downfall of PCR is the presence of PCR inhibitors in urine and the formation of crystals when urine samples are preserved at low temperature [5]. Recombinase polymerase amplification (RPA) allows for the amplification of CT directly from patient samples [6]. The RPA detection method for CT involves a 90℃ incubation for 5 minutes(heat treatment of urine) for DNA extraction and 10 minutes for amplification at 38℃, but based on test results, specificity is recorded at 83% [6]. Although the turnaround time decreased by up to 50% with the use of RPA compared to PCR dependent techniques, a much higher specificity must be attained for clinical use.

Microwave-accelerated metal-enhanced fluorescence (MAMEF) technique has been used to lyse CT bacterial DNA with low level microwave exposure, and silver island films have been proved as a reliable method of visualizing the effectiveness of microwave lysing [7]. Although MAMEF itself can be used to detect CT with tethered complementary silver nanoparticles within seconds, the time to process and amplify DNA from a patient sample impedes the rapidity of the entire test.

Experimental Methods: Primer design must not only incorporate modifications necessary for detection on lateral flow strips, but also for gold nanoparticle aggregation. As per design by Krölov group, CDS2 is conserved in most CT strains, and primers were designed to be 30-35 nucleotides long with pyrimidines at the 5' end and purines for the last three sequences in the 3' end which serves as a stable target for the polymerase during RPA [6]. The primers that were determined from the experiment were
Forward 5′-CCTTCATTATGTCGGAGTCTGAGCACCCTAGGC-3′ and

Reverse 5′-CTCTCAAGCAGGACTACAAGCTGCAATCCCTT-3′ which targeted CT CDS2 and possessed either a 5’ Biotin or FAM modification for detection on lateral flow strips [6]. Following the required primer design, gold nanoparticles are conjugated as follows:
Forward 5′-CCTTCATTATGTCGG-au-3’ 5’AGTCTGAGCACCCTAGGC-au-3’ and
Reverse 5′-CTCTCAAGCAGGACTAC-au-3’ 5’AAGCTGCAATCCCTT-au-3′.
Highly conserved sequences of GAPDH(human housekeeping gene) are also used to create primers with similar 5’ modifications as a positive control for the RPA assay. For the GAPDH primers, there are no conjugated gold particles since it will not produce a discernible color change(i.e. A color change of GAPDH cannot be determined separately from CT since both are present in one sample). Thus, the primers for GAPDH are the same as the primers discussed by Krölov:
Forward 5’-AAGTCAGGTGGAGCGAGGCTAGCTGGCCCGATT-3’ and
Reverse 5’-CACCATGCCACAGCCACCACACCTCTGCGGGGA-3’

CDS2 Primers are split into two separate sequences with gold nanoparticle modifications on both the 3' ends. Additionally, gold nanoparticle design must also be considered to produce a distinct color change before and after aggregation. Varying parameters such as the shape and size of a gold nanoparticle [8] creates a color change from yellow(before aggregation) to blue(after aggregation). Experiments must first take place to determine the best combination of shape and size of gold nanoparticles to maintain the specificity of lateral flow strips, while presenting the user with contrasting color changes. To determine the overall function of primer design and aggregation color change, the CT test is carried out with DNA extracted samples as opposed to direct patient samples. Improvement of design for a more robust primer may be necessary if the primers fail to detect CT in urine samples. An additional factor for a more reliable assay may be to test the results of CDS2 detection with one or two bp deletions and how aggregation is affected with such deletions.

After collection of a urine sample, the sample is heated to 90℃ for the lysate preparation phase and RPA-au primers are added to the sample. During this period, RPA-au primers may bind and the test will present a visual color change or lack thereof, depending on the presence of CT target DNA. Afterward, the RPA cycle continues with amplification and CT detection follows the protocol of lateral flow strips of the Krölov group. To measure the specificity of the RPA-au primer proposal method, tests must be made with a group of patients willing to participate in the study.

Material Characterization and Expected Results: Although the proposed method does not serve as a complete detection method in itself, it essentially serves as a harbinger for the presence of CT DNA. When CT DNA is present, both RPA-au primers will bind which will allow for a change of color from yellow to blue. A sample that has natural yellow pigment may possibly change to a green color. If there is a color change during the incubation phase, and the results of the lateral flow strips are congruent(CT positive), then it is under the assumption that the patient is positive for CT. However, if there are conflicting results(e.g. If there is no color change upon the addition of RPA-au primers, but the results of lateral flow strips display CT+, there may be an expected false positive). The gold nanoparticles yield a result that can be characterized by a photospectrometer, but the intended results should be visibly discernible in hopes to decrease turnaround time. Efforts to create drastic changes in color upon gold-nanoparticle aggregation is emphasized to bypass the use of a photospectrometer. Essentially, the proposed method allows the user to make conclusions based on an “If-and-then” approach that relies on both the proposed method and the conventional lateral flow strip tests. (e.g. If there is no color change and there are both bands on the lateral flow strips, then it is a false positive). During the lateral flow strip CT detection, a CT positive test is

expected to produce two bands. One band for GAPDH, which ensures the functionality of RPA, and a second band that indicates the presence of CT.

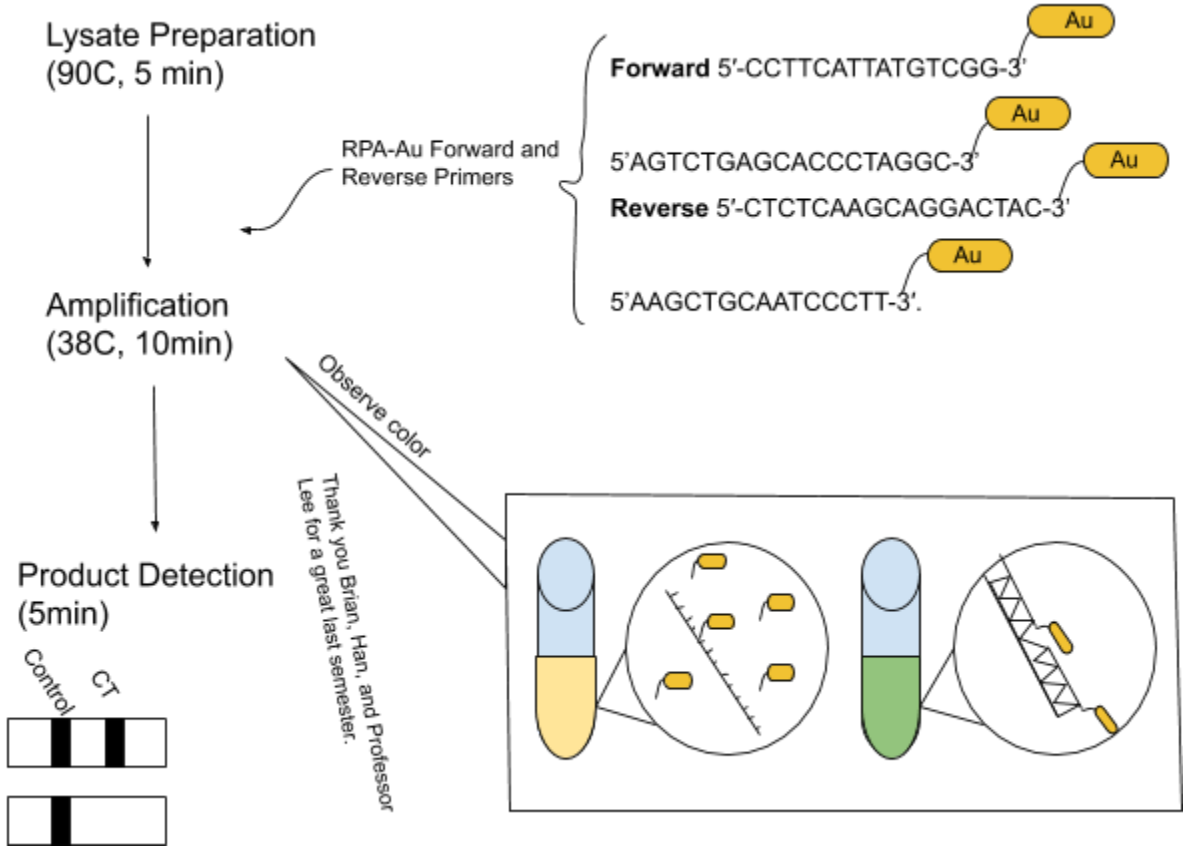

Figure 1. Illustration of proposal and principle mechanics. RPA-Au forward and reverse primers are added to solution. Binding of RPA-Au primers result in aggregation of gold nanoparticles and a color change from yellow to green. Lack of target sequence will result in no color change.

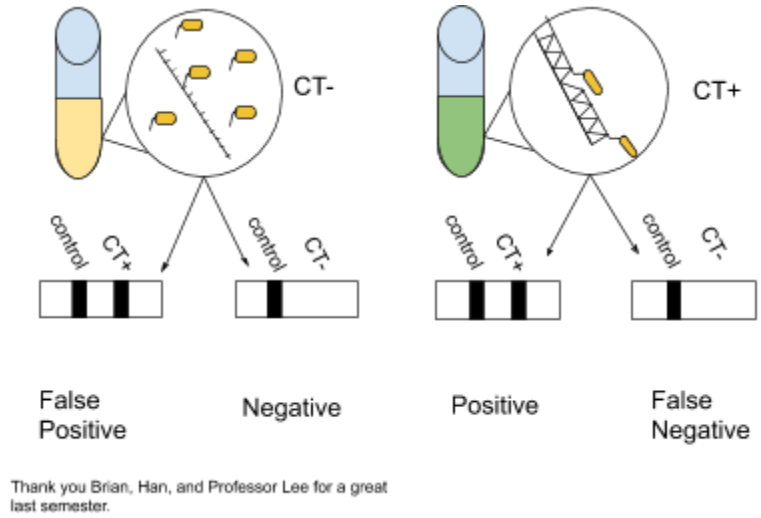

Figure 2. Expected results from the addition of gold nanoparticle conjugated to RPA primers. On the left, solution color remains unchanged since there are no target sequences. On the right,

solution color changed to green which indicates the presence of target sequence. Continuation of the test with lateral flow strips either confirms or contradicts the solution litmus test.

Conclusion and Future Direction:

Overall, the proposed gold-nanoparticle conjugated RPA primers serve as a seamless secondary screening technique to determine false positives and negatives in CT tests while providing a rapid turnaround time of ten minutes. Paired with lateral flow strips, the proposed gold-nanoparticle assay produces clear results that may contradict or confirm the results of lateral flow strips to ultimately improve specificity.

Although the proposed CT rapid detection test aims to provide a quick turnaround time with RPA from patient samples, there must be a plethora of experiments to improve the accuracy and robustness of the RPA-au primers: Determination of the size/shape of gold particles and the optimal primer design. The seamless integration of the secondary screening technique aims to improve specificity for bacterial STDs; future attempts and applications should be made to improve the specificity of other bacterial STDs such as Gonorrhea and Syphilis. Furthermore, emphasis must be placed on the developments to integrate the proposed screening technique in saliva tests.

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