Optimization of the NEB LAMP Test - Project 163: https://app.jogl.io/project/163

Summary of Work to Date
17 May 2020

This project was funded by JOGL and AXA Research Fund in Round 1. Funds were deposited 23 April, 2020 and therefore week 0 is considered to have begun 23 April. Week 8 would conclude 17 June. However, we have already made progress in all of our proposed milestones within 4 weeks of receiving funding.

Original Round 1 Project Timeline.
See comments below for an explanation as to what has been completed.

- Week 0 - Order reagents (primers, enzymes, buffers, positive control DNA/RNA)

- Week 1 - Modifications of ratio of primers and magnesium concentrations to assess maximal efficiency of the reaction and its detection. Order synthetic RNA positive controls.

- Week 2 - Test relating to buffers and treatments for the sample transfer/processing/ use using synthetic RNA controls

- Weeks 3 & 4 - Final Validations of Sensitivity and Specificity with Controls (by day 28)

- Week 5 - Write up data and transfer methodology to partner lab with BSL2 facilities (CDC partner lab and possibly others)

- By Week 8 - Conclusions (Is this method robust enough for dealing with samples in this pandemic?) and broad dissemination of the open information.

Evaluation of LAMP Primer Sets
To date, we have evaluated four LAMP primer sets:

1) "Zhang N-A" https://www.medrxiv.org/content/10.1101/2020.02.26.20028373v1
   This primer set targets the viral N-gene and is referenced in the above pre-print and is linked directly from the NEB website where the LAMP WarmStart Colorimetric Master Mix can be purchased. The first and last authors work directly for NEB.

2) "Mammoth N" https://mammoth.bio/wp-content/uploads/2020/03/Mammoth-Biosciences-A-protocol-for-rapid-detection-of-SARS-CoV-2-using-CRISPR-diagnostics-DETECTR.pdf
   This primer targets the viral N-gene and is referenced in the above pre-print. The protocol has been developed by Mammoth Biosciences.

3 & 4) "NEB N2" + "NEB E1". These primer sets are unpublished. They target both the viral N-gene and E-gene. The sequences were provided directly by Nathan Tanner at NEB, who told us they are more sensitive than the Zhang N-A primer set. These primer sets were evaluated separately and together in LAMP reactions. Tanner also recommended adding 40mM guanidine HCl to the LAMP reactions.

Results: We were able to detect SARS-CoV-2 using all four of the above primer sets. However, NEB N2 + NEB E1 together are the most sensitive with an LoD between 10-50 viral copies across three labs (more tests will be run). Positive results are as early as 20 minutes, while negative control samples stay negative for 60 minutes. Optimal conditions were incubation at 68 degrees C (versus the standard 65 degrees C), 2X concentration of the Loop primers and the addition of 40 mM guanidine hydrochloride, pH 8. The Mammoth N-gene primer set performed the worst, with many false positive results for negative controls. The Zhang N-A primer set performed well, but was not as sensitive (LoD ~ 300 viral copies) as the NEB N2 + NEB E1 sets.

Evaluation of Positive Controls for SARS-CoV-2

After initiating this project, data were published questioning the concentration information provided by the suppliers of some of the controls (e.g. the IDT plasmids). Therefore a variety were tested.

To date, we have evaluated 5 positive controls for SARS-CoV-2:

1) Synthetic N-gene DNA positive control plasmid from IDT: https://www.idtdna.com/pages/landing/coronavirus-research-reagents
2) Synthetic RNA control from Twist: https://www.twistbioscience.com/resources/twist-synthetic-sars-cov-2-rna-controls
3) Heat-inactivated virus in *Vero* cell culture from Zeptometrix: https://www.zeptometrix.com/products/sars-cov-2-isolate-usa-wa1-2020-culture-fluid-heat-inactivated-05-ml
4) Heat-inactivated virus in human epithelial cell culture from ATCC via BEI Resources: https://www.beiresources.org/Catalog/antigen/NR-52350.aspx

5) Extracted viral RNA from ATCC via BEI Resources: https://www.beiresources.org/Catalog/BEINucleicAcids/NR-52347.aspx

Results: We were able to detect all five of these positive controls for SARS-CoV-2 using the NEB LAMP colorimetric assay.

Evaluation of Cross-Reactivity for Closely Related Coronaviruses

To date, we have evaluated cross-reactivity for 2 positive controls for other closely related coronaviruses:

1) Synthetic N-gene DNA positive control plasmid for SARS-CoV from IDT: https://www.idtdna.com/pages/landing/coronavirus-research-reagents
2) Synthetic N-gene DNA positive control plasmid for MERS-CoV from IDT: https://www.idtdna.com/pages/landing/coronavirus-research-reagents

Results: We found no cross-reactivity for either the positive control for SARS-CoV or the positive control for MERS-CoV.

Next Steps:

Evaluation of Sample Collection Methods, Buffers and RNA Concentration/ Purification Methods Using Spiked Synthetic Clinical Samples

Since this project began, there have been several publications that investigate the number of virus particles in samplings via swab or by collection of fluid such as saliva. Saliva is the easiest fluid to collect, but studies suggest that the number of particles per mL, particularly when diluted in inactivation medium, is too low for direct addition to LAMP to be meaningful in all cases. This is also the case for swabs placed in media such as Viral Transport Media. So, although the LAMP reaction appears to be more rugged than RT-qPCR with regard to interference from sample collection media and the sample components, the most reliable test will probably have to include an RNA extraction step. Saliva is an attractive method to collect samples, because it is non-invasive and there are commercial collection kits that would facilitate home collection. The kit contains inactivation medium and the resulting tube can be mailed safely. The RNA would then be extracted from the tube. Besides purifying the RNA away from potentially interfering components, an extraction step also concentrates the RNA and therefore renders the test more sensitive.

To date, we have been optimizing the LAMP reactions mostly using purified nucleic acids (synthetic DNA plasmids, synthetic RNA and extracted RNA). We believe we now have optimized primer sets using NEB N2 + NEB E1 and optimized temperature of 68 degrees C, plus 2X concentration of the Loop primers and the addition of 40 mM guanidine hydrochloride. We have begun to explore the creation of synthetic clinical samples. In addition, since existing Covid-19 tests always include a positive control for human RNA, we will begin assessing control primer sets to make

sure we can detect human cell RNA as a positive control that our LAMP test is working properly and the sample actually contained material from a patient.

We have begun preliminary tests adding a dilution of the raw Zeptometrix virus in *Vero* cell culture (not extracted) directly to LAMP reactions and have positive detection. We have also spiked saliva with dilutions of the raw Zeptometrix virus in *Vero* cell culture, then extracted viral RNA using a commercial kit (E.N.Z.A.), and we have positive detection.

We have identified several published extraction methods to compare, and are aiming to develop a method that is inexpensive, efficient, and does not depend on backordered reagents. So far we have identified the following methods as promising, and will test them first with spiked synthetic clinical samples.

1) iron bead-based protocol (Hillbilly beads)
2) silica (glass milk) from Harvard paper
3) Syringe filter protocol

Validation Using Actual Clinical Samples in Partnership with Hospitals
-Partners TBD

The final phase of lab work will be to test the performance of the optimized method from sample collection to result using actual clinical samples and compare it to the results using a validated method such as RT-qPCR. This will entail partnerships with access to clinical samples. We are now establishing contacts and have several possible partners, including Chiris Mason at Weill-Cornell Medical Center, the CDC, and international partners.