Field-ready genetic coronavirus test for use in low-resource underserved populations

Background: Due to increases in climate change, deforestation, and human population, viral pandemics are predicted to become more frequent.[1] Key technological advances in testing are needed to limit the impact of pandemics on human mortality and global economies. Testing for active infection allows early identification of carriers, both symptomatic and asymptomatic, isolation of affected areas, thus limiting spread. From an individual health care perspective, testing allows differentiation of the pandemic disease from diseases with related symptoms to guide proper treatment regimens. Broad and efficient testing would also allow community activities to continue in a safer environment and avoid the negative consequences of job loss, risk of exposure to infected coworkers while on the job, reduced educational opportunities, and social isolation.

Most current testing technologies rely on sequence identification using nucleic acid isolation, thermal cycling, and detection of fluorescence markers using expensive advanced technologies. These types of tests are only available in areas where a developed and coordinated medical infrastructure is present. For example, a leading testing approach in the United States is the Abbott test[2], which uses a proprietary all-in-one Abbott PCR thermocycler and detection apparatus (approximate cost = $60,000) resulting in a cost per assay of $40-60 (cost billed to insurance). These approaches also require skilled technicians to run the assays.

Advances in testing that make it more accessible would benefit the current health disparities associated with the current COVID-19 pandemic. Demographics show that minority populations are bearing a disproportionate number of COVID19 cases and deaths, both in the U.S. and in other countries.[3] This disparity represents a combination of medical and socioeconomic factors. Undoubtedly, health disparities will be associated with future pandemics, regardless of the underlying biological mechanisms, due to socioeconomic limitations such as access to healthcare, more crowded living conditions, lack of ability to work from home, and significantly, limited access to expensive testing.

As one response to the health disparity issues, we envision an efficient point-of-care assay that can be used in underserved communities to test for active carriers of coronavirus. We propose to establish a streamlined, inexpensive process for extracting nucleic acid that is of sufficient quality and quantity for immediate use in an isothermal PCR reaction. We are confident in the success of the project because all components/steps required for the project have been established or have previously been shown to be feasible. In this proposal, we describe a path to develop a novel SARS-CoV-2 test which can be run at the point-of-care (e.g., rural developing nations, and underserved portions of the United States). The test would be nucleic acid-based and thus adaptable to other pathogens, such as pandemic flus (recent example, the H5N9 influenza strain recently detected in China).

Specific Aim: **Instrument-free nucleic acid template extraction and analysis**. Human saliva will be spiked with heat-inactivated SARS-CoV-2 (ATCC, BSL1). Nucleic acid will be harvested by mixing spiked saliva with lysis reagents followed by centrifugation to separate nucleic acid sample from insoluble fraction. Nucleic acid integrity will be evaluated by traditional qPCR and isothermal PCR for SARS-CoV-2 sequences. PCR products will be detected by fluorescence (later goal will be to covert to visual read-out). *Success Criteria/ Go-No Go milestone: Isolation procedure optimized to produce intact template and both qPCR and isothermal PCR to confirm that quality and quantity is sufficient for practical testing (copy number at or above 100)*. **There are multiple features of this test which could provide the basis for IP protection once validated.**

Experimental Plan

Our overarching goal is to develop a streamlined, field-ready genetic test for coronavirus in low-resource regions. To optimize the genetics-based strategy, a field-ready test that can be performed with results interpreted by any lay person is critical. The test would have an easy-to-interpret visual read-out (such as available now in a pregnancy test). Since the test would be easy to implement and inexpensive, testing could be done multiple times during the course of a pandemic without hardship. Moreover, since specificity is driven by primer choice, the assay can easily be adapted to other viral pathogens that will arise,

Our approach uses technologies that have all been independently validated (no new technology breakthroughs are needed) but have not been combined into one system (**Figure 1**). The test involves, 1) Sample Collection: Isolation of biological specimen (saliva) in lysis buffer, 2) Sample Preparation: separation of a nucleic acid-containing fraction, 3) Isothermal PCR: mixture of pre-loaded lyophilized assay reagents, and 4) Detection of Amplified Product: employ colorimetric dye for visual read-out.

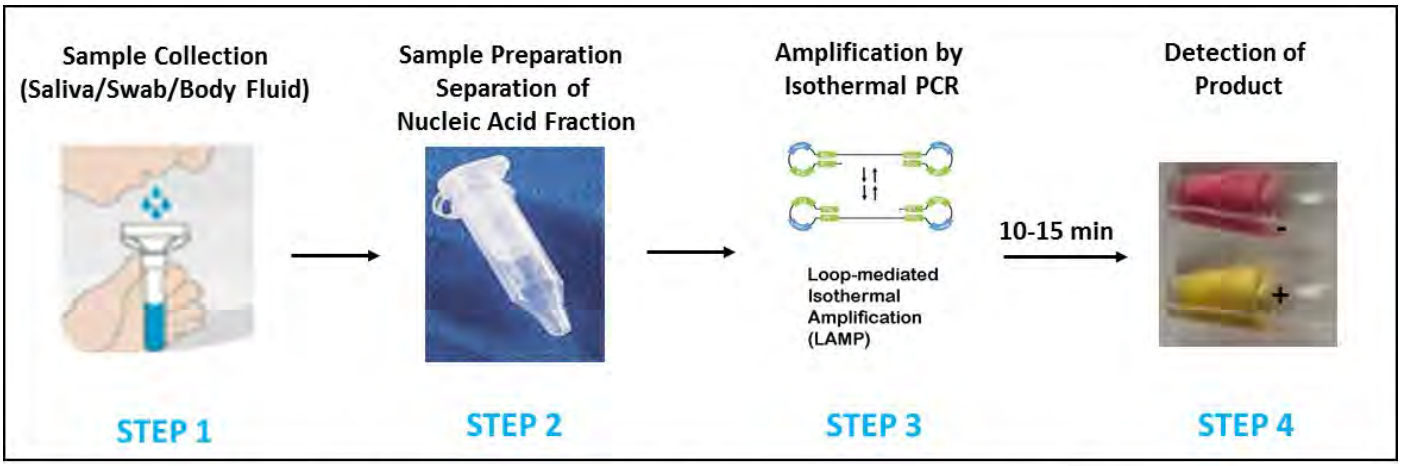

Figure 1: Schematic Representation of Coronavirus Field Test

This proposal will focus on the first 3 steps in the process:

Step #1 Sample collection:

Fig. 2 Collection of human biological specimen. Plastic collection apparatus for saliva or mucus collection will be used.

The foundation of this project is isolation of genetic-analysis-ready nucleic acid from a human sample (initially saliva) with minimal processing. The key components desired for initial mix are the nucleic acid extraction buffer and the specific primers and enzymes needed for isothermal PCR. We have identified a commercially available plastic collection tube for saliva. Saliva can be collected into a tube containing a lyophilized lysis mix, thus keeping volume minimized for greater sensitivity (dilution of samples into larger volumes, such as during Abbott test, decreases sensitivity and may be one factor in the high false-negative rates observed in these assays). Sample collection vials are labeled with fill lines to standardize collection volumes. Rutgers has developed and is using a similar saliva-collection vial in their coronavirus testing development for patients with active virus infection, both symptomatic and asymptomatic (https://www.nj.com/coronavirus/2020/05/a-spit-test-for-the-coronavirus-developed-by-rutgers-should-soon-be-available-for-use-at-home.html). Saliva will be the initial starting material, but mucosal samples may also be tested in future tests.

Step #2 Sample Processing: We will process biological specimen by A) adding to optimized lysis mix, and B) heating using a heat-block (future optimization for field work may employ hand held warmers), and C) separating out soluble nucleic acid fraction using a standard lab centrifuge (future optimization for field work may employ

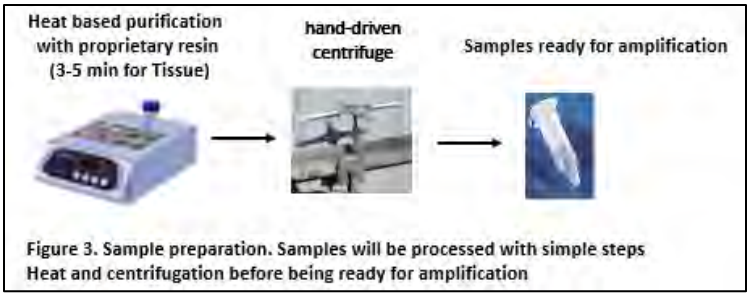

Figure 3. Sample preparation. Samples will be processed with simple steps
Heat and centrifugation before being ready for amplification

hand-powered[4] or battery powered mini-centrifuge) (**Figure 3**). Samples would be contained in collection vial with safety screw cap and rubber gasket. For hand-held or battery centrifugation to be investigated in the future for field applications, the centrifugal forces would be well below forces that would risk tube rupture. Samples would be inside plastic enclosure during the spin. In this proposal, we will use standard centrifuge with a lid in a lab setting with trained personnel using heat-inactivate virus.

From previous work performed by our expert consultant on this project, we have already developed analogous methods in previous studies where samples were processed for traditional qPCR assays (unpublished data). In these studies, a preservation solution that allows for easy collection and processing of saliva for a direct qPCR without the requirement of nucleic acid extraction was develop which gave equivalent results to nucleic acid purified by more extensive traditional laboratory methods (unpublished data, see **Figure 4**). Given this experience, we do not foresee any problems developing a similar assay reagent that facilitates isolation of nucleic acid for isothermal PCR.

This method allows a one tube procedure without repetitive operation. To assess the efficiency of viral RNA recovery, we will use standard RT-PCR to quantitate the amount of viral nucleic acid present. The procedure itself will subsequently be adjusted for use in isothermal PCR.

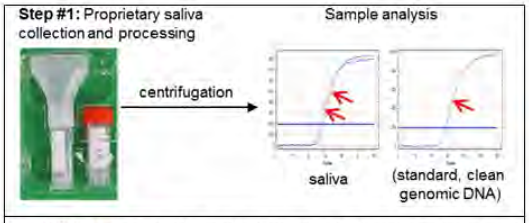

Fig. 4 Saliva for genetic tests. Example of previous collection and processing of saliva suitable for direct PCR amplification of genes of interest. Sample quality validated using PCR amplification (results from two reactions are shown) which is comparable to those obtained from the high purity genomic DNA prepared following a standard laboratory procedure.

Steps #3 and #4 Isothermal PCR and Detection of PCR Products: (*Step 4 to be carried out in future studies after this proposal is completed*). The idea is to perform PCR at room temperature or fixed one temperature, giving the advantage of machine free and fast (no cycling). Technology advances in isothermal PCR make this goal more readily achievable.[5] We will detect the product with Sybr-Green, which from our previous studies, has been shown to be more sensitive and faster than the real time PCR protocols currently established by the Centers for Disease Control for detecting SARS-CoV2. Reverse transcription of viral RNA to DNA and amplification of DNA will be performed in one step. Isothermal primers will be designed using primer explorer (https://primerexplorer.jp/e/) and multiple primers will be screened to identify a set of 6 primers for the ORF region of the viral sequence. Preliminary data has proven the effectiveness of primers for both qPCR (CDC protocol recommended primers) and isothermal amplification. It took about 30 min to detect viral sequence in a traditional qPCR reaction and take 5-15 min for the signal of isothermal PCR (data not shown). A similar strategy will be applied for screening of a primer set targeting the N region of the virus. In this study, inactivated virus RNA will be tested for one step detection. We will gather data on assay reliability regarding experimental false positive and false-negative rates in blinded studies using virus-free saliva versus samples spiked with heat-inactivated virus. We will also use spiked samples and RT-PCR to determine the limit of detection. Future work will investigate sensitivity and reliability (real-world false positive and false negative rates) using samples from human volunteers. Viral detection limit is currently around 100. With our plan of concentrating the viral extract during processing, we expect to lower the detection limit to below 10 copies.

In the future, we will optimize lyophilized reagents for stability and easy storage,[6] and the readout will be converted to colorimetric[7], which will be read by the naked eye. The temperature range that allows effective amplification will be determined, although we expect the polymerase-mediated reaction will be effective in temperatures that are typical in the areas where test will be used. Alternatively, heating pads or hand warmers might be used to avoid need for electricity.

In summary, all technologies described in the proposal are available or have been shown to be feasible. The final test components will be readily assembled into a kit for field work (**Figure 5**) with a price point at pennies per assay. The convenience of operation, increased sensitivity and adjustable throughput (low to medium

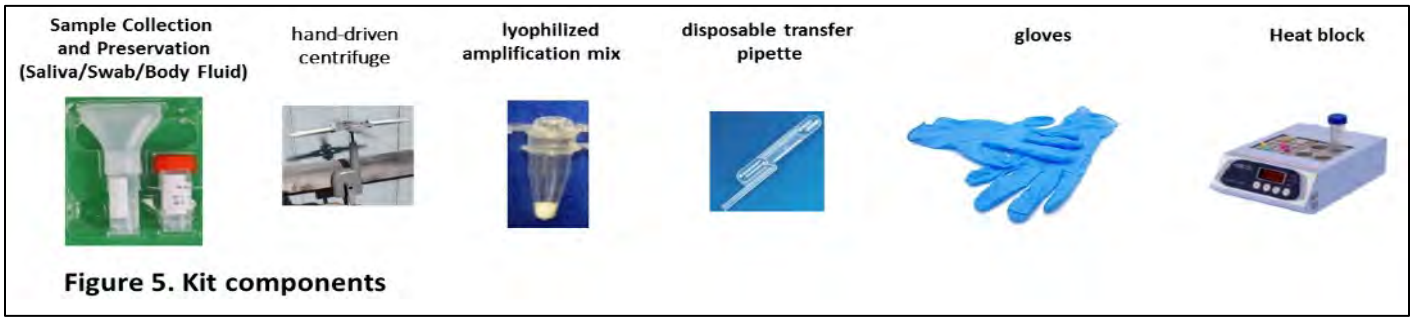

Figure 5. Kit components

throughput) of our assay allows health workers to perform these assays without extensive training in a low setting environment for a more accurate diagnosis of the disease. Our eventual goal is to assemble low cost all-in-one disposable devices for viral nucleic acid detection for convenient personal use. In the future, if the need for coronavirus tests diminishes, the technology advances we make in these proposed studies can be quickly applied to another pathogen, in medicine or in agriculture.

Competitive landscape: Most new technology efforts in coronavirus testing are being performed by large companies that sell expensive equipment in parallel to support their assay (e.g., Abbott ID now assay) and the throughput for these devices is typically low. To date, no at-home or field test assays are available. FDA authorized home tests so far are limited to home-based sample collection, not testing. Thus, there are opportunities for point-of-care assays. Efforts in this area by other companies have been reported, for example by Bosch Healthcare solutions, but our process is different from others that have been reported. The advantages/disadvantages for each coronavirus detection assay versus competitors can only be evaluated at a later stage of development. We have a distinct advantage in having the experience and knowledge of Dr. Kumar who has successfully developed a isothermal-based test for tuberculosis and FangPing Zhao who has spent years developing nucleic acid-based diagnostics.

Timeline (6 months)

	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
Step #1 Sample collection: test and optimize range of SARS-CoV2 spiked lysates using standard centrifuge and qPCR as read-out	■	■				
Step #2 Sample processing: Test hand or battery powered centrifugation methods			■	■		
Step #3 Isothermal PCR: Compare results in lysate samples between traditional qPCR and isothermal PCR					■	■

Budget:

Lysate reagents - standard laboratory salts and detergents (Sigma-Aldrich)	$1,000
Disposables/plasticware (Fisher)	$2,000
Heat-inactivated SARS2-CoV-2 (ATCC, 2 vials)	$1,400
qPCR primers and probes (IDT)	$5,000
qPCR Reagents with fluorescent probes(NEB)	$4,000
Isothermal PCR reagents with fluorescent prbes (NEB)	$9,000
Research Scientist, Fangping Zhao (see Biosketch)	$18,000
Total	$40,400

Plan to apply for future funding:

Preliminary data from this 6-month project would allow us to spin-out a new company (with aid from NCCU Office of Technology Transfer, Dr. Undi Hoffler) and apply for NSF SBIR/STTR funding. **Several steps in the process would provide opportunities for patent application.**

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