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14. ABSTRACT

Mesa Photonics developed a simple and fast test for detecting SARS coronavirus in infected cells. The test system consists of substrates that release fluorescent signals when they are cleaved by a SARS and a control protease. The fluorescent signals are detected simultaneously in one sample in a battery-operated handheld fluorometer. The SARS reporter substrate is not cleaved by human proteases or proteases from other coronaviruses. We demonstrate that 15,000 viruses can be detected in less than 6,000 cells in 15 minutes using crude, unpurified lysate.

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Final Report

Phase I SBIR Project

SBIR Phase I Project Title: A New, Simple, Rapid, On-Site Test for Detection of and Prevention from SARS-CoV Viruses

Performing Organization:

Mesa Photonics, LLC

1550 Pacheco Street

Santa Fe, New Mexico, 87505

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1 Contents

2 Summary

Mesa Photonics is reporting on the results of its DoD SBIR Phase I project in which we demonstrate a new approach to testing for the presence of SARS coronavirus. The test measures the activity of a SARS-specific protease enzyme in infected cells with a reporter substrate that is recognized only by SARS coronaviruses. The specimens tested are tongue scrape samples that subjects can collect themselves. The test is fast (less than 30 minutes), easy to use, rapidly deployable, scalable, and has a low logistics burden for military logisticians, clinics, medical treatment facilities, frontline workers, and forward deployed military and civilian personnel.

The Phase I project was a success. We demonstrate multiplexed detection of recombinant PLpro and the "built in" control, endogenous Angiotensin-Converting Enzyme 2 (ACE2), in lysates from tongue scrape cells. We confirmed specificity of our PLpro reporter substrates: **a)** it is not cleaved by proteases naturally present in tongue scrape lysates and **b)** it is not cleaved by NL63, the only other coronavirus known to infect human cells via the ACE receptor. The multiplex test was successfully transferred to an existing, battery-operated, handheld fluorometer. The instrument was configured so that both substrates can be measured in the same sample. A multi-sample tester was conceptualized that Mesa Photonics will further develop in Phase II.

The New Mexico Small Business Association (NMSBA) awarded Mesa Photonics $20,000 in-kind for technical assistance from Los Alamos National Laboratories to further validate the approach in cultured coronavirus-infected cells. Testing was performed in a Biosafety Level 2 laboratory using human coronavirus strains 229E, OC43 and NL63, which express papain-like proteases that are similar to SARS PLpro. The viruses were infected into tissue-cultured cells, the cells lysed and the test performed using 4 different substrates. The PLpro reporter substrate of Mesa Photonics test showed high specificity: it was not cleaved by NL63, OC43 or 229E. ISG15 was cleaved after 15 minutes in lysates from cells infected with 229E and OC43 whereas ubiquitin was cleaved after 15 minutes in lysates from NL63 infected cells. This result corresponds to the ubiquitin substrate preference observed using recombinant NL63 papain-like protease. To our knowledge, this is the first report that the coronaviruses 229E and OC43 have the ability to cleave ISG15. Minor OC43 cleavage of the tetrapeptide with natural amino acids was observed after 60 minutes.

Coronavirus protease activity was detectable in as little as 6,000 cells that were infected with 15,000 viruses. In comparison, seven current antigen tests required $2x10^6$ – $2x10^7$ viral copies for detection,[1] corresponding to over 100 times more copies than our test requires. Although RT-PCR is the most sensitive analytical test, it suffers from high false-negative rates due to rapid degradation of the RNA sample in clinical applications.[2] In contrast, our biomarker is stable and our tongue scrape collection method is expected to deliver samples with highly concentrated virus: the tongue is estimated to have 30 e^6cells/cm^2 of which ~ 1% are ACE positive. Each cell can be infected by 10-100 viruses[3]; thus, we expect to harvest 300,000 ACE positive tongue cells which could contain between 3 million to 30 million viruses, which is around 200 times more than our test requires.

The high sensitivity and stability of our test greatly reduces the uncertainty of success for our test system performing in human samples. We believe that the outcome of Phase I provides a strong foundation for further increasing the sensitivity of our SARS PLpro test in Phase II, for obtaining regulatory approval, and for commercializing test reagents and fluorometers.

3 Impact

The test has several important applications for SARS diagnostics and therapeutics:

1) Rapid, simple, on-site diagnostics test: Widespread screening of populations has been hindered by high false-negative rates of RT-PCR and antigen tests which are further increasing due to emerging variants. Our portable, simple, and affordable SARS-CoV-2 test system is predicted to be a "**one-for-all**" solution for detecting SARS wildtype and variant strains with applications for Point of Care (POC) and on-site settings. Customers include forward deployed personnel, airlines, border control, refugee camps, businesses, schools, colleges, and homes. The test is simple, rugged and battery-operated and readily deployable, for example by parachuting, to remote locations.

2) Direct measurement of blocking activity: Emerging SARS variants are predicted to evade the blocking effect of antibodies that are generated by vaccines and/or natural immune responses or are administered as therapeutics. Annual vaccines will therefore likely be needed and their efficacy in blocking viral entry determined in clinical trials. Our approach is the only one that can directly determine the blocking effect of anti-spike antibodies by measuring "escape variants" that were able to enter cells. Immediate customers of our test reagents are researchers developing agents designed to bock virus from entering host cells. Potential customers of our portable diagnostic test are pharmaceutical companies and labs developing new vaccines and/or therapeutic antibodies that require clinical trials. Enrolled subjects would be provided with Mesa's portable test system in form of a companion diagnostics test with which they can take measurements at home and transmit the data via a phone app to the clinical trial center.

3) Cell-based drug screening: drugs that can inhibit the activity of SARS enzymes and thereby prevent viral replication are promising future therapeutics. Our assay is suitable for high content screening in crude cellular lysates which closely reflect the natural environment of the cell. Potential immediate customers are research groups involved in high content or high throughput screening.

Future applications. The test platform can be used to detect other human pathogens (e.g., other viruses and bacteria) and pathogens affecting animals. For example, Foot and Mouth Disease (FMD) is a severe, highly contagious viral disease of livestock that has a significant economic impact.

4 Background

4.1 Coronaviruses

Three global epidemics of coronaviruses have so far emerged in this century.[4] In 2002, infections with Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) were reported in China. Ten years later the Middle East Respiratory Syndrome coronavirus (MERS-CoV) appeared in Saudi Arabia and spread worldwide.[5] In December 2019 a novel coronavirus—Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2)—was identified in Wuhan, China.[6,7] Current studies indicate that this coronavirus is similar to SARS-CoV-1.[8] The main symptoms of these coronavirus infections are similar to influenza-like complaints and include fever, headache, malaise, shivering and diarrhea. SARS-CoV-2 also infects the lower respiratory tract (bronchial tubes and the lungs) where it can trigger an inflammatory response as the body produces antibodies and T cells to fight the virus. Around 15% of people experience an overreaction of their body's immune system, called a cytokine storm.[9] This can severely damage the lungs, kidney and heart and cause clot formation, causing some people to become severely ill or die.

With a fatality rate of ~2.3%, SARS-CoV-2 is much less fatal than SARS-CoV-1 (9.5%) or MERS (34.4%).[10] However, because initial symptoms of COVID are benign, the virus spreads more easily among asymptomatic carriers who can unknowingly spread the virus before they experience symptoms.

Coronaviruses enter cells through specific attachment to receptors on the host cells. The Middle East Respiratory coronavirus (MERS) enters cells via the DPP4 receptor, 229E via CD13, HKU1 and OC43 via sialic acid. SARS-CoV-1, SARS-CoV-2 and N63 coronaviruses enter cells by binding to the specific host cell receptor ACE2, which is highly expressed on epithelial cells of the gastrointestinal tract, lung and the oral cavity.[11,12]

The processes involved in SARS-CoV entry into cells and production of Mesa Photonics biomarker, PLpro, is illustrated in Figure 1. The figure illustrates an important feature of our test: our biomarker PLpro is present only in host cells that are infected with live virus. In contrast, RT-PCR and antigen tests cannot differentiate between dead or live viruses that are inside or outside of a cell.

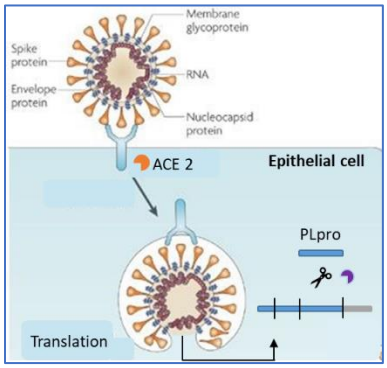


Figure 1. SARS infection. The spike proteins of SARS-CoV-1 and SARS-CoV-2 bind to ACE2 receptors on epithelial cells. The cell membranes fuse, the virus enters the cell and releases its mRNA that is translated by the host cell machinery to produce several proteins. One of those is PLpro that is released from a polypeptide chain through autocleavage. PLpro cleaves 3 sites with amino acid motif LXGG. Our reporter peptide substrate is based on this sequence. The more benign NL63 coronavirus also enters cells through ACE2 receptors but does not cleave our reporter substrate, as demonstrated in Figure 12.

Mesa Photonics test is based on detecting fluorescence that is released from two peptides following cleavage. SARS coronavirus infection is detected using a synthetic, short protein fragment (peptide) with an amino acid sequence identified by Rut et al (2020)[13] that PLpro from SARS, but not MERS, recognizes. In the uncleaved substrate the fluorescence is quenched by the electron-withdrawing properties of the amide bond that connects the last amino acid with the fluorophore aminomethyl coumarin (AMC). When PLpro cleaves the amide bond a fluorescent signal is generated, as shown in Figure 2.

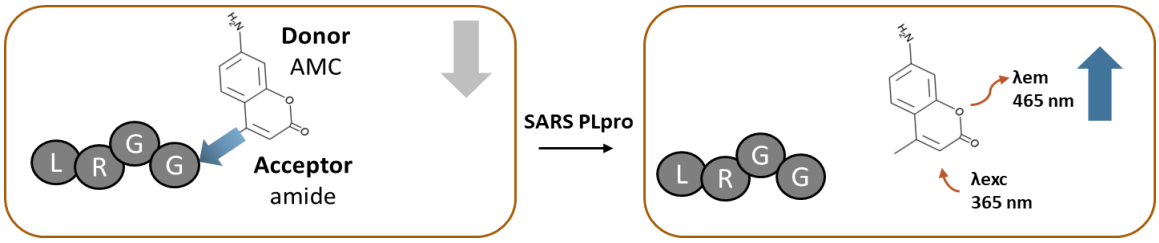


Figure 2. Schematic of Mesa Photonics PLpro assay. A synthetic peptide (gray circles) is labeled with the donor fluorophore aminomethylcoumarin (AMC). The amide bond between the last amino acid and AMC withdraws electrons from AMC, and fluorescence is quenched. When the peptide is cleaved by SARS PLpro, quenching no longer occurs and the AMC fluorescence is released (right, blue arrow).

The second peptide—the control—produces a fluorescent signal at a different wavelength than the PLpro peptide. This peptide is recognized by human angiotensin converting enzyme 2 (ACE2) that is ubiquitously expressed on tongue epithelial cells through which SARS coronaviruses enter cells.[7] The control confirms that the sample collected has sufficient quantity and quality for the test to perform properly. Both peptides are measured simultaneously in one sample. This peptide is labeled with a quencher on one end and a fluorophore on the other. Photons emitted by the fluorophore upon excitation by a light source (e.g., mercury lamp or LED) are absorbed by the quencher that "turns off" fluorescence. The energy transfer from the fluorophore to the quencher is highly distance dependent and occurs only up to a distance of approximately 10 nm (corresponding to approximately 28 amino acids). Therefore, when the quencher and fluorophore move apart when the peptide is cleaved, fluorescence is "turned on" (Fig. 3).

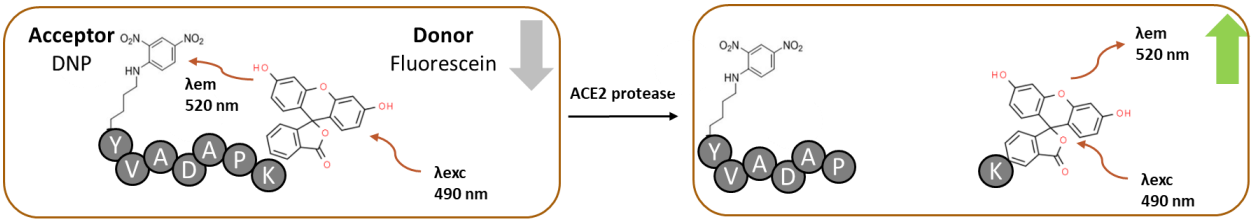


Figure 3. Schematic of ACE2 protease assay. Left. A synthetic peptide (gray circles) is dually labeled with a donor fluorophore (fluorescein) and an acceptor (dintrophenyl, DNP). **Right**. When the peptide is excited, energy is transferred from the donor to the acceptor. When the peptide is cleaved, the donor and acceptor move apart and fluorescence is emitted from the donor fluorophore.

The PLpro reporter substrate and the ACE2 control peptide are read simultaneously at different wavelengths in one detection vial.

One advantage of these enzyme assays is that the proteases are not consumed in the cleaving process. They are available to continue cleaving sites until all sites have been cleaved, effectively amplifying the signal. This process is evident in the data by the slow, but steady increase of fluorescence over time.

4.2 Mesa Photonics Testing Approach

In Phase I, fluorescent peptides were combined so that PLpro and ACE2 activities were detected simultaneously in one sample. The assay was adapted to a commercial, battery-operated handheld fluorometer and will be performed as shown in Figure 4.

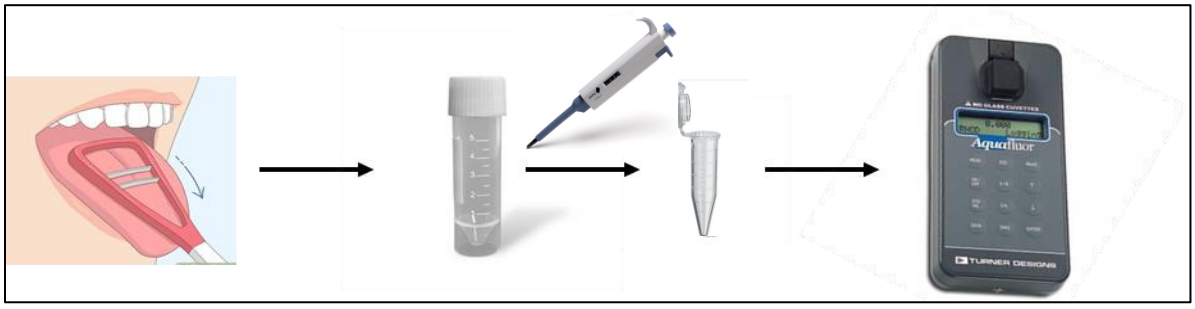


Figure 4: Overview of Mesa Photonics SARS test and components. A tongue scrape sample is collected and deposited in a specimen vial containing a lysis buffer that disrupts the lipid membrane of cells and viruses. The solution is transferred to a vial that contains all components for the test in a freeze-dried, and therefore stable, form. The cuvette is inserted into a battery operated, handheld fluorometer that has 2 channels: one to measure emission from the positive control ACE2 peptide and the second to measure emission from the PLpro peptide. The channels are switched by a simple push of a button.

Measurements are taken immediately and after 15 minutes. The difference in fluorescence units is calculated and the results interpreted as follows: A fluorescence increase in both the PLpro and the ACE2 channel indicate SARS infection (pos), a positive signal in ACE2 the absence of infection (neg) and the absence of fluorescence increase in the ACE2 channel indicates an inconclusive result (inc).

PLpro	+	-	+/-
ACE2	+	+	-
	pos	neg	inc

A commercial kit will contain a tongue scraper, a vial with lysis buffer, a single channel pipettor for transferring the sample to a 0.5 ml tube containing all test components in freeze-dried, and therefore stable, form. The vial is transferred to simple battery-operated fluorometer. Test cuvettes and sampling material for single tests will be commercially available for < USD 20 and sold separately from the reader. Test reagents will also be sold in bulk for research purposes or for high content screening. The current reader is expected to be available for ~ USD 3,000. It is battery-operated, waterproof, and ruggedized, making it ideally suited for quick decontamination and parachuting into remote locations.

5 Comparison of Mesa Photonics Protease test relative to the state of the art

5.1 SARS-CoV-2 real time RT-PCR

The current "gold standard" for SARS-CoV-2 testing is RT-PCR. The technique employs several primers with sequences that are complementary to the target cDNA, and in addition a probe that binds specifically to a region of the SARS-CoV-2 DNA that is being amplified. The probe is labeled with a quencher and a fluorophore that fluoresces when the polymerase cleaves the probe to remove it from the DNA strand. The amount of fluorescence correlates with the amount of DNA being produced and can be observed in real time.

The Centers for Disease Control and Prevention (CDC) and other government health agencies developed a real-time reverse transcription PCR (RT-PCR)[14] test after the SARS-CoV-2 genome sequence was shared with the global scientific community through an online resource. Based on this sequence, primer and probe sets were developed that bind specifically to three regions of the COVID gene sequence.

RT-PCR is in principle able to detect a single copy of genetic material. It is the most sensitive and accurate test in terms of **analytical** sensitivity, i.e., when performed on isolated and purified RNA. However, RNA degrades easily during shipment and purification, and sampling and handling can fail so that the **clinical** sensitivity of RT-PCR is much lower. Indeed, the false-negative rate is at least 20%, and depending on the viral load at the time of testing can be 100%[15] as shown in Figure 5.

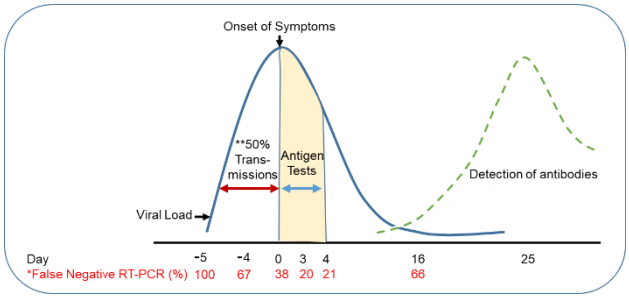


Figure 5. RT-PCR false negative rates. RT-PCR has false-negatives rates (x-axis, red) between 100% to 38% in the five days (x-axis) before symptoms appear, when 50% of transmissions occur. Antigen tests are not sensitive and detect viral protein only within the 5 days after symptom onset. Antibodies against SARS can be detected only 16-25 days after infection. Serology tests therefore cannot detect active infections.

5.2 SARS-CoV-2 antigen tests

Antigen tests are typically cheap and return results in minutes. They detect the presence of viral proteins in a biological sample, such as a NP swab. Generally, drops of the sample are spotted on a surface, such as a plastic well or a test strip, that is coated with one antibody that binds the SARS protein. Another antibody detects bound protein. If both antibodies bind, a signal is generated, such as a color change, that can be read visually although some setups use small readers to improve the accuracy.

The challenge with antigen tests is finding antibodies that both bind to the single viral protein such as the SARS-CoV-2 spike protein at separate sites but do not cross react with other coronaviruses or bind nonspecifically. Weak signals are a major drawback because antigen tests do not amplify the protein signal; therefore, they are inherently less sensitive than RT-PCR or enzyme assays. As a result, most antigen tests require 10,000 to 100,000 viral copies for detection.[16]

In addition to a high false-negative rate, antigen tests share some of the disadvantages of RT-PCR tests, namely that they can detect inactive virus and may no longer bind when viral antigens are mutated.

5.3 Mesa's test is less susceptible to false-negatives from variants

SARS-CoV-2 has now been estimated to have infected over 130 million people worldwide.[17] This huge reservoir of replicating viruses has an enormous potential for developing mutant strains to which primers, probes and antibodies bind less efficiently or no longer at all. Indeed, four RT-PCR-based tests have already been flagged by the FDA: Curative SARS Covid RT-PCR test, Accula SARS-Cov-2 Test (Mesa Biotech now Thermo Fisher), TaqPath COVID-19 Combo Kit (Thermo Fisher), Linea COVID-19 Assay Kit (Applied

DNA Sciences).[18,19] More are expected to follow. Although RT-PCR tests amplify cDNA from several regions of the SARS genome that generally do not all fail at the same time, one variant in France was altogether undetectable by RT-PCR.[20] But even the absence of only one PCR product triggers an "inconclusive" result and the analysis must be repeated by a different test or sequencing. This is not practical in most settings.

In contrast, mutations in our SARS biomarker PLpro are rare because mutations in enzymes or their cleavage sites generally lead to viral loss of function.[21] For this reason, we believe that our test can be a "**one-for-all**" test that can reliably detect infections across variants.

5.4 Direct detection of infected cells: application as companion diagnostics test

The majority of antibodies produced by the body as a result of infection or vaccination are directed against the SARS spike protein. This protein is rapidly mutating, resulting in escape variants that can efficiently infect cells in people who were thought to be immune due to antibodies generated in previous infections or by vaccinations. For example, in Manaus, Brazil, a study of blood donors indicated that 76% of the population had been infected with SARS-CoV-2 by October, 2020, yet were re-infected with SARS-2 variants later.[22] Booster vaccines against variants are already in development. Although booster vaccines will not have to complete full FDA approval as the first vaccine generation, in which generally around 30,000 subjects are enrolled, Phase I clinical trials will be necessary (as is the case with annual flu vaccines) that will include ~ 300 subjects.

Because Mesa Photonics biomarker PLpro is produced only in, and by, infected cells and is not present outside of cells in virions, our test is the only one that can directly measure how efficiently a vaccine or antibody is able to prevent viral entry into a cell. RT-PCR and antigen tests cannot distinguish between viral material that is inside or outside of cells. Therefore, our test has great potential not only as an R&D tool for vaccine developers, but also as a companion diagnostics test for use in clinical trials for FDA authorization. Subjects could take daily measurements of viral load and send the test results directly to the clinical research organization, e.g., via a phone app.

Indeed, AstraZeneca has expressed great interest in and support for our test as a companion diagnostics tool in a CLIA-waived setting.

In comparison to the state of the art, Mesa Photonics results demonstrate that our PLpro test has several important advantages that are summarized in the table below.

Table 1: Comparison of features between PLpro protease, RT-PCR and antigen tests

Feature	**PLpro protease**	**RT-PCR**	**Antigen**
Clinical Sensitivity	**~ 6000 cells**	1000 – 10,000	10,000-100,000
False negative rate	**tbd**	20-100%	20-100%
False positive rate	**tbd**	High – residual virus	High - residual virus
Live/dead virus	**Live**	Live or dead	Live or dead
Detection in cells	**Yes**	No	No
Training	**Minimal**	Specialized personnel	Minimal
Time to reporting	**<30 minutes**	days	<30 minutes
Specimen	**Tongue scrape**	Nasopharyngeal/saliva	Nose swab
On site testing	**Yes**	No	Yes
Scale up	**Synthetic substrate easy to scale up**	Biological Taq polymerase; already at capacity	Antibody scale up limited
Cost per test	**USD 10 - 20**	USD 100	USD 10 - 20
Cost for instrument	**USD ~ 3,000**	USD 15,000-90,000	USD 5,000 (optional)

6 Phase I Accomplishments

The Phase I project was designed to answer the following key questions:

(a) Can we perform the PLpro test together with the ACE2 test in one reaction, in other words can we multiplex?

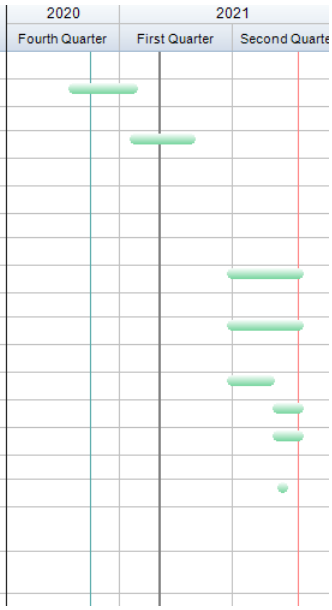
(b) What changes to the assay protocol are needed for the test to perform in a battery-operated handheld fluorometer?

(c) What engineering design do we need to build a multi-sample tester in Phase II?

The questions the Phase I project was designed to address were answered. The work done in Phase I builds a sound foundation for the development of a prototype test system in Phase II of the project that will be validated, clinically tested, and for which emergency use authorization will be submitted.

An overview of the project is shown below.

Task	Duration (Days)	Resources Assigned	Start Date	Finish Date	% Complete
Task 1 - Multiplex					
Multiplexed PLpro/ACE2 test in lysates	35.00	FHR	11/24/20	1/11/21	100%
Task 2 - Adapt to portable spectrometer	33.00		1/13/21	2/26/21	100%
Multiplexed test in Turner Designs instrument					
Configure instrument channels					
Task 3 - Multisample Tester					
Design drawings of prototype	38.00	DJK	4/1/21	5/24/21	100%
Additional Work funded by NMSBA	38.00	LANL	4/1/21	5/24/21	100%
In vivo testing in lysates from cells infected with					
229E coronavirus	22.00		4/1/21	4/30/21	100%
NL63 coronavirus	12.00		5/7/21	5/24/21	100%
OC43 coronavirus	12.00		5/7/21	5/24/21	100%
Invited presentation resulting from this work	1.00	FHR	5/11/21	5/11/21	100%
CLEO Super Symposium on Photonics Solutions for COVID-19 Challenge III					
Title: ENZYME ACTIVITY DIAGNOSTICS of SARS INFECTION BASED on FOERSTER RESONANCE ENERGY TRANSFER					



We consider each of the Phase I Objectives now and describe how we accomplished them.

6.1 TASK 1 Multiplex PLpro/ACE test in lysates

6.1.1 Optimize PLpro assay

Recombinant PLpro (BPS Biosciences) and substrate (LRGG-AMC) were diluted in assay buffer (80 mM Tris, 1 mM DTT, pH 8.0) and 5 µl of each added to wells of a 384-well white plate. The final concentrations of enzyme and substrate were 20 nM and 50 µM, respectively. A suite of detergents was purchased from Thermo Fisher (Surfact-Amps Detergent Sampler) and each detergent diluted to 10% with ddH_20. 5 µl of each substance to be titrated was then added to duplicate wells and the plate read at λ_{exc} = 345 nm and λ_{em} = 445 nm in a plate reader (Molecular Devices). The delta RFU between wells with and without enzyme was calculated for each substance. Figure 6 shows the optimal concentration of each variable tested.

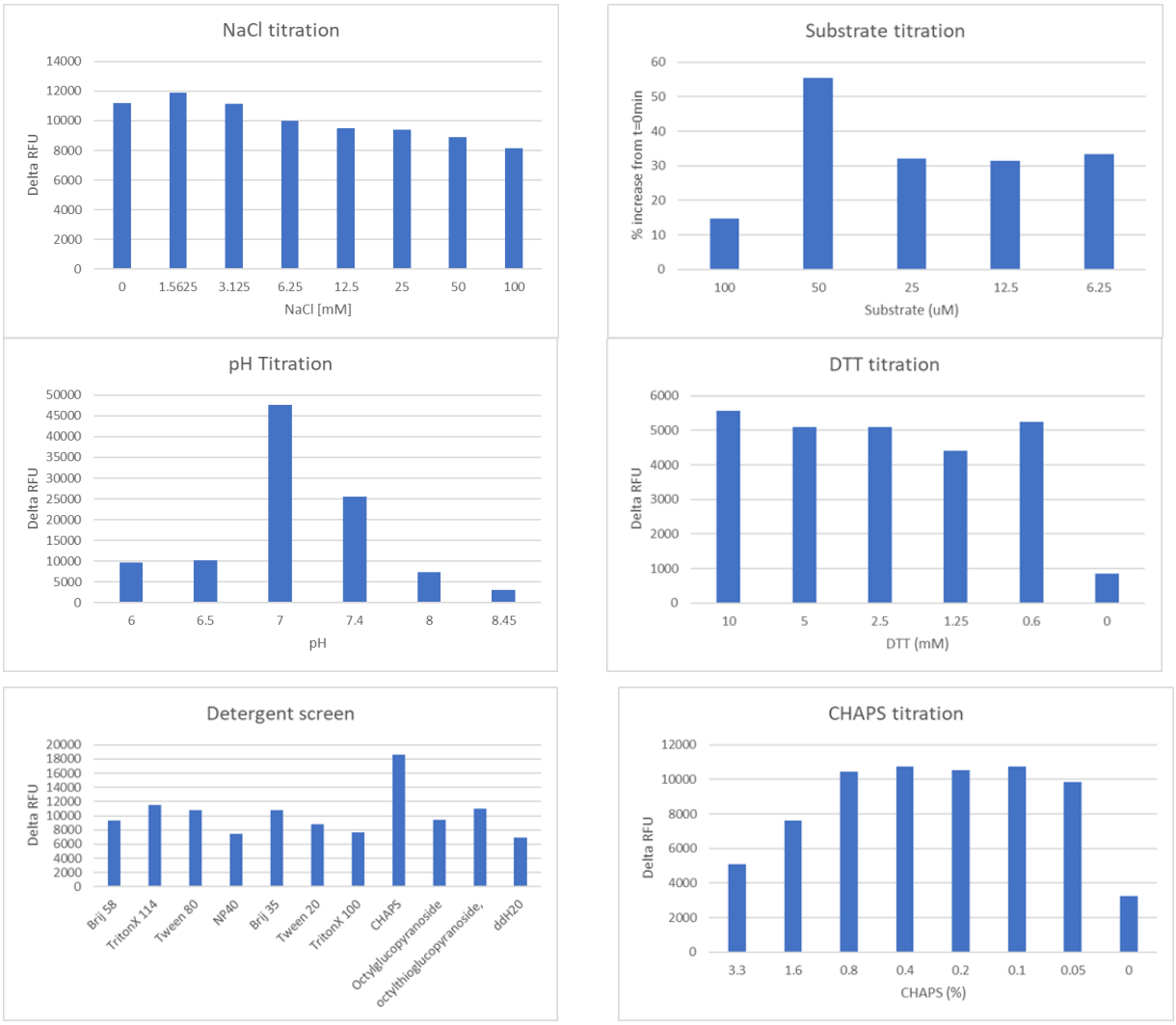


Figure 6: Assay Buffer Optimization. Wells of a 384-well plate contained substrate (50 µM) and PLpro enzyme (20nM) or no PLpro. The delta RFU between wells with and without enzyme were calculated in the presence of the component to be tested. The highest delta indicates the preferred concentration.

Conclusion: the optimal assay buffer for PLpro is 40 mM Tris, pH 7.0; 0.1 % CHAPS; 2 mM DTT. A 65-fold improvement in the assay performance was achieved with this buffer compared to the one reported in the literature.[23]

6.1.2 Test PLpro substrates with different fluors

Rationale: Rut et al reported that a PLpro substrate with the sequence hTyr-Dap-Gly-Gly-ACC is specifically recognized by SARS-1 and SARS-2 but not by MERS coronaviruses or human proteases.[13] We reasoned that because TAMRA is spectrally distant from the ACE2-AMC labelled substrate we used at the time, we could generate our multiplex with a PLpro substrate with the sequence Q1-hTyr-Dap-Gly-Gly-TAMRA. This substrate was custom synthesized by Cambridge Scientific.

Approach: The conversion of this substrate was compared to that of the PLpro substrate Z-LRGG-AMC.

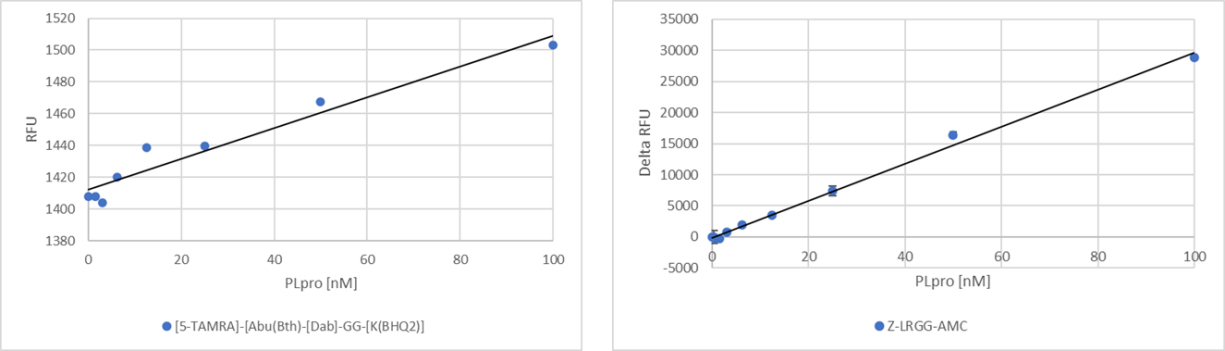


Figure 7. Conversion of different PLpro substrates. Various concentrations of PLpro were added to wells of a 384-well plate in the presence of 20 µM TAMRA-labeled (left) or AMC labeled (right) PLpro substrate.

Result: The TAMRA substrate is very poorly converted (delta ~ 100 RFU compared to ~ 30,000 with Z-LRGG-AMC), which is likely due to sterical hindrance in the substrate binding site.

Conclusion: Instead of changing the validated SARS PLpro substrate with the ACC fluorophore and the amino acid sequence hTyr-Dap-Gly-Gly, we used an ACE2 substrate labeled with spectrally removed FITC.

6.1.3 Multiplex PLpro and ACE2

Rationale: Performing the SARS PLpro reaction together with the positive control in one vial reduces complexity and cost of the test. The commercially available FITC-labeled ACE-2 substrate was tested for its performance as a multiplex substrate in combination with the PLpro substrate hTyr-Dap-Gly-Gly-ACC.

Approach: The ACE2 substrate FITC-YVADAPK-DNP was purchased from Bachem and tested for performance in tongue scrape lysate (Figure 8).

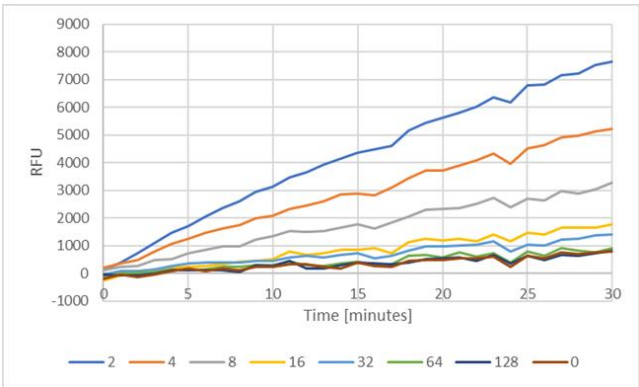


Figure 8. ACE2 substrate performance in tongue scrape lysate. Serial dilutions of tongue scrape lysate were prepared in lysis buffer and combined with 1 µM FITC-ACE substrate in 2x PLpro reaction buffer. The reaction progress was monitored over 30 minutes using $\lambda_{exc/em}$ 490nm\520nm in a fluorescence plate reader.

Result: The peptide is converted well in PLpro assay buffer. The signal roughly titrates with the lysate concentration.

Conclusion: The FITC-labeled ACE2 substrate is a good multiplex partner for the ACC-labeled PLpro substrate. The spectral features of the multiplex substrates are shown in Figure 9.

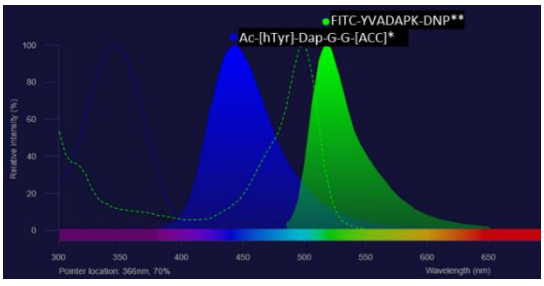


Figure 9. Excitation and emission spectra of the proposed PLpro/ACE2 multiplex pair. The excitation (dotted line) and emission (filled peak) spectra for ACC are shown in blue and those for FITC in green.

6.1.4 Validation of the multiplex pair

Rationale: The separation of ACC and FITC emission spectra appears sufficient for the proposed multiplex but must be validated experimentally.

Approach: FITC-ACE2 and ACC-PLpro substrates were combined in wells and the fluorescence compared with that from reactions that contained only one substrate.

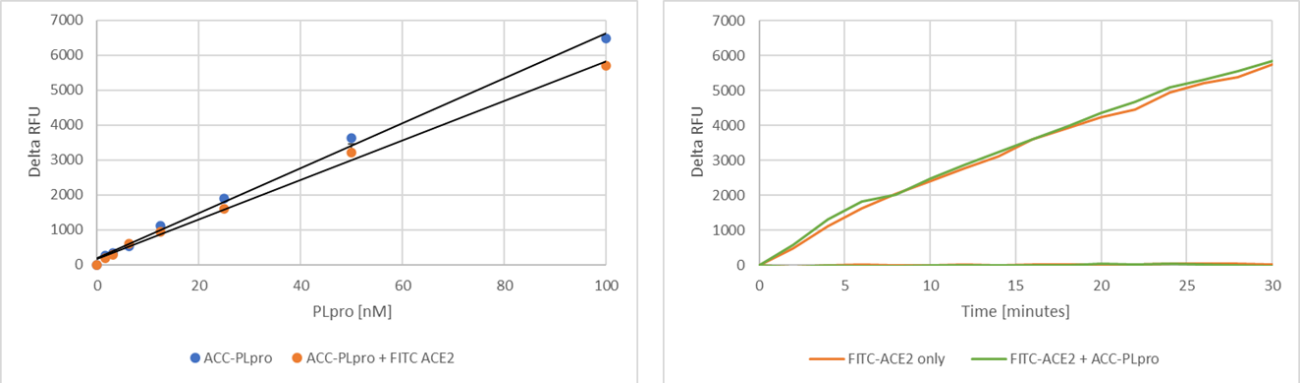


Figure 10. Multiplexed versus singliplexed reactions. Left: Various concentrations of PLpro were added to wells of a 384-well plate containing either ACC-PLpro substrate alone (blue circles) or ACC-PLpro substrate + FITC-ACE2 substrate (orange circles). The reaction was monitored after 30 minutes incubation at room temperate using λexc/em wavelengths of 345/445 nm. Right: Tongue lysate was added to wells of a 384-well plate containing either FITC-ACE substrate only (orange trace) or FITC-ACE substrate plus ACC PLpro substrate (green trace). The reaction was monitored in real time for 30 minutes using λexc/em wavelengths of 490/520 nm.

Result: There is no appreciable difference in the performance of reactions containing one or both substrates.

Conclusion: The multiplex pair ACE2-FITC and ACC-PLpro is validated.

6.1.5 Specificity Testing

Rationale 1: Rut et al report some cleavage of the PLPro substrate with the sequence LRGG-AMC by human endogenous deubiquitylating enzymes, whereas human DUB enzymes and MERS do not cleave the substrate with unnatural amino acids [h-Tyr]-[Dap]-GG-ACC.[23] The absence of SARS PLpro substrate cleavage by human deubiquitylating enzymes in tongue scrape lysate was to be confirmed.

Approach: The substrates were added to fresh tongue scrape lysate and the reaction progress monitored.

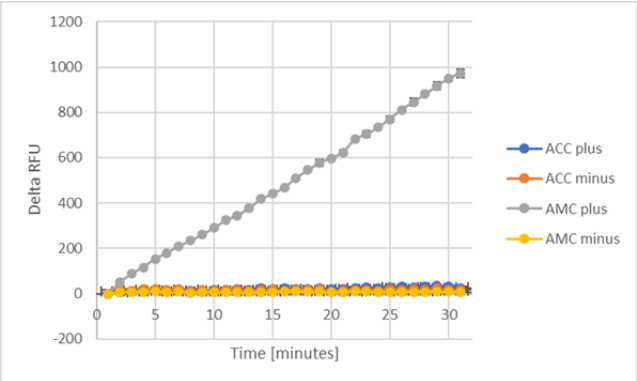


Figure 11. No cleavage of Mesa's reporter PLpro substrates in tongue scrape lysate. Substrates with the sequence LRGG-AMC and [h-Tyr]-[Dap]-GG-ACC were added to wells of a 384-well plate containing fresh tongue scrape lysate (plus) or assay buffer (minus). Only substrate with the amino acid sequence LRGG is cleaved in lysate (grey).

Result: Endogenous human enzymes present in tongue scrape lysate do not non-specifically cleave the [h-Tyr]-[Dap]-GG-ACC substrate, the substrate Mesa plans to use in its test.

Rationale 2: The more benign NL63 coronavirus also enters cells via ACE2 receptors and could be detected with our test instead of SARS. It is reported that NL63 PLpro cleaves the substrate LRGG between the two glycines[15] so that fluorescence remains quenched by the electron-withdrawing properties of the amide bond between the last glycine and AMC (see Figure 2). This was to be confirmed.

Approach: Recombinant PLpro from NL63 was purchased from BPS Biosciences and its ability to cleave our PLpro substrate tested.

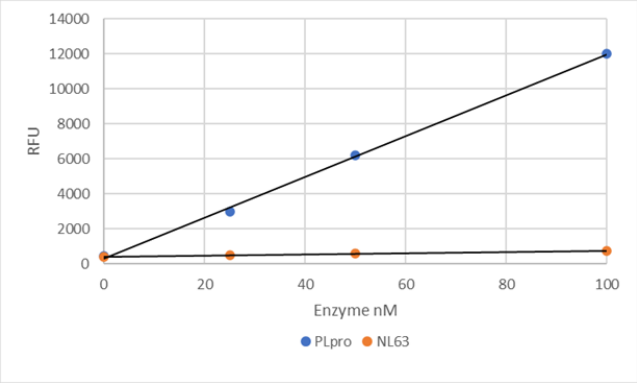


Figure 12. NL63 does not cleave Mesa's reporter substrate. Serial dilutions of recombinant PLpro from SARS (blue circles) or NL63 (orange) were added to wells containing 30 µM [h-Tyr]-[Dap]-GG-ACC substrate. The reaction was performed for 30 minutes at room temperature and measured in a plate reader using λexc/em wavelengths of 345/445 nm.

Result: Our PLpro test substrate is cleaved only by PLpro from SARS and not by NL63.

Conclusion: detection of NL63 coronaviruses with out test are not expected.

Task 1 was successfully completed.

6.2 TASK 2–Adapt test from a plate reader to a commercial handheld fluorometer.

6.2.1 Compare reactions in different volumes

Rationale: Now that the reaction conditions and spectral configurations for optimal performance of our multiplex pair were determined, we moved to testing the performance using volumes and wavelengths that are compatible with the commercially available Aquafluor instrument from Turner Designs.

The reactions in the plate reader were performed in 15 µl – 20 µl volumes, whereas measurements in the Turner instrument require a volume of at least 80 µl. In addition, the Turner instrument measurement settings differ slightly from those used in the plate reader.

Approach: PLpro reactions that were performed in different volumes and with different measurement parameters were compared.

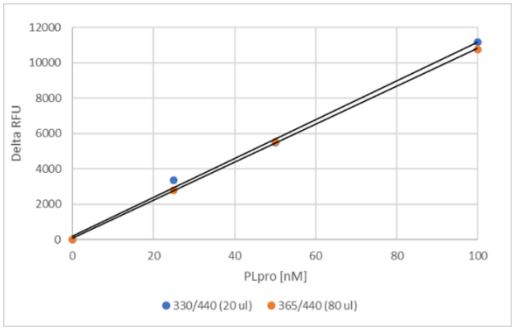


Figure 13. Comparison of PLpro assays between plate-based instrument and handheld instrument settings. Various concentrations of PLpro were added to wells of a 384-well plate for identical final concentrations in 20 µl and 80 µl volumes. Substrate was added to achieve a final concentration of 20 µM and wells monitored using plate-based instrument settings (blue) or the Turner instrument channel settings (orange).

Result: There is no appreciable difference in the performance of enzyme in different volumes and when measured at different wavelengths.

Conclusion: We are ready to test the assay in the Turner handheld instrument.

6.2.2 Perform Multiplexed ACE2/PLpro Test in handheld fluorometer

Approach: A handheld fluorometer was purchased from Turner Designs with channels configured for multiplexed measurement. Individual and multiplexed ACE2 and PLpro reactions were measured (Fig. 15).

Problems encountered: (1) The small volume cuvettes that were originally proposed for measurement in the handheld device did not fit into the cuvette port of the instrument. Instead, a cuvette adapter was purchased that accommodates 0.5 ml PCR tubes. (2) In the original channel configuration, the emission from the FITC substrate was detected in the PLpro-AMC channel. The problem was solved by reconfiguring the emission filter. The final configuration is as follows:

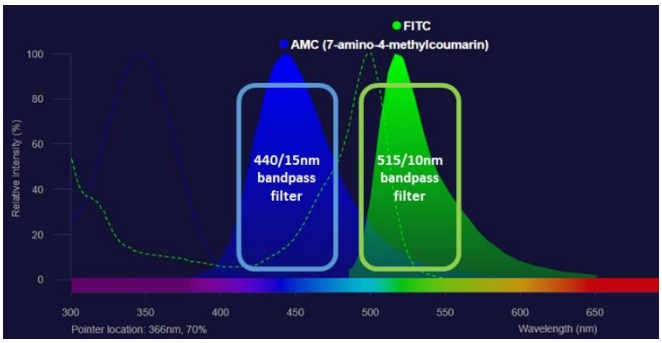
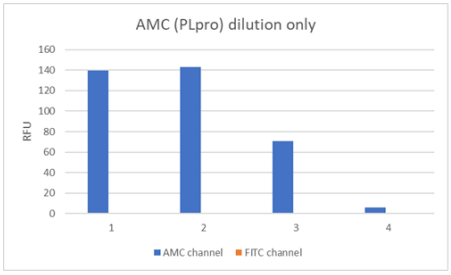
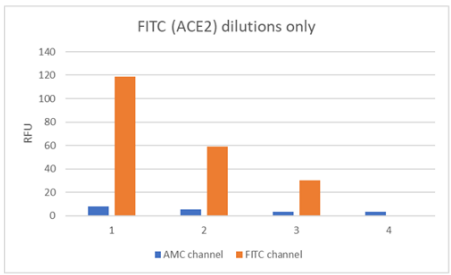


Figure 14. Configuration of the optical parameters in the handheld device.
Channel A (PLpro-ACC; blue)
LED: UV
Excitation filter 365 nm
Bandpass filter 440/15nm
Channel B (ACE2-FITC; green)
LED Blue
Excitation filter 475 nm
Bandpass filter 515/10nm





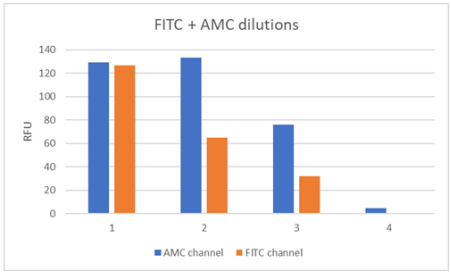


Figure 15. Multiplexing in the Turner handheld instrument.

A) Recombinant PLpro (100 nM, 50 nM, 25 nM, 0 nM) was combined with PLpro-AMC substrate and the relative fluorescence measured in the AMC and the FITC channels (orange).

Result: There is no interference from the FITC channel (not visible in this graph).

B) Three dilutions of fresh tongue scrape lysate were combined with the ACE-FITC substrate and the relative fluorescence measured in the AMC and the FITC channels.

Result: The signal from the AMC channel is negligible.

C) Reactions A and B were combined in one vial and the fluorescence measured in the AMC and the FITC channels.

Result: The relative fluorescence units measured in the multiplex are comparable to those obtained from the single measurements in A and B.

Conclusion: The multiplex was validated in the handheld instrument.

Task 2 was successfully completed.

6.3 TASK 3 – Design Multi-Sample Tester

Rationale: Mesa Photonics is preparing for the commercial need of measuring multiple samples by designing its own multi-sample tester.

Approach: Major optics design features that are to be included in the instrument have already been identified through our Phase I work (see Fig. 14).

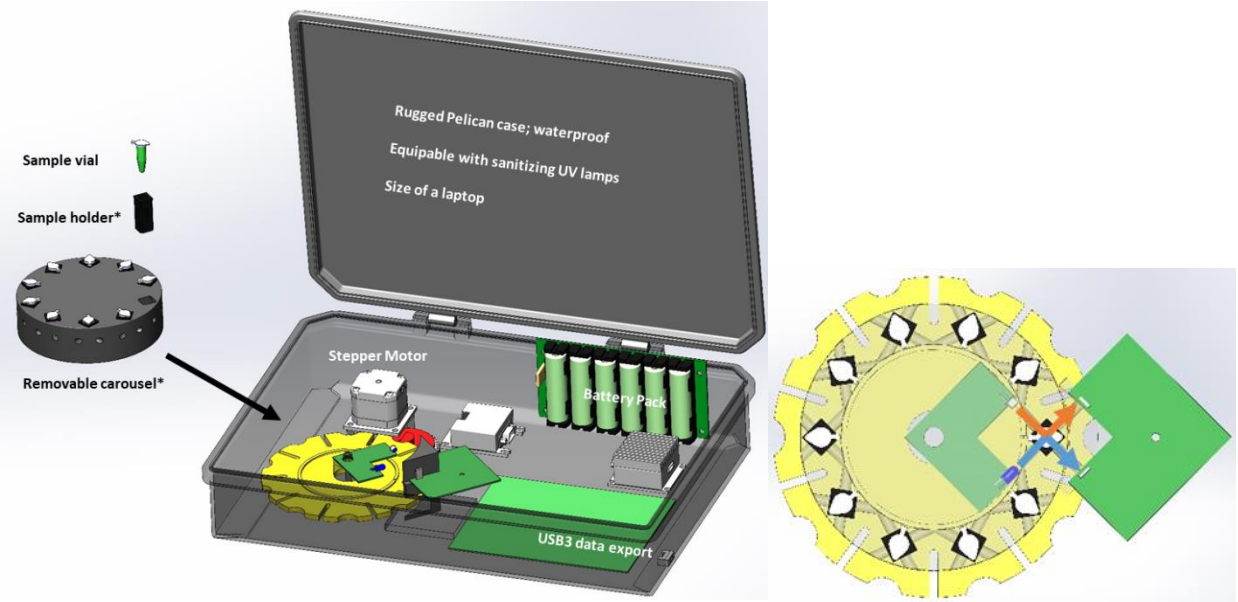


Figure 16. Preliminary Design of a multi-sample tester. **Left.** Samples are collected in a sample vial and placed in a sample holder that is inserted into a carousel. The carousel is then placed onto a Geneva wheel (yellow) which is driven by a stepper motor past the excitation sources. **Right.** Two light-emitting diodes deliver excitation light at different wavelength (blue and orange arrows) and pass through the samples inserted in a carousel. The emission is detected by two photodiodes arranged on the other side of the Geneva wheel (white dashes on green board). * parts designed to be 3D printed.

The enclosure depicted on the left is approximately 11.5 in X 16 in X 4 in. The case can be sealed and ruggedized, making it compatible for delivery to remote locations. The lid of the case can be equipped with UV-C bulbs for easy sterilization. The instrument contains a USB3 data export port from which raw data can be exported to a phone app or to a computer.

Task 3 was successfully completed

7 Other Collaborating Organizations

Los Alamos National Laboratory provided technical assistance (in-kind contribution amounting to 20K) through the New Mexico Small Business Assistance Program (NMBSA) to validate the test on the "benign" coronavirus strains 229E, OC43, and NL63. These strains are similar to SARS PLpro and express papain-like proteases, but they have not all been characterized for their ability to cleave substrates with the LXGG motif of our test.

For NL63 and SARS, it is known that this amino acid motif is cleaved in the commercially available ubiquitin substrates UbiQ-AMC and ISG15-AMC with a higher catalytic rate than is seen with the tetrapeptide. Recombinant papain-like protease from NL63 (but not from 229E and OC43) is commercially available and we therefore first determined the NL63 activity against UbiQ-AMC, ISG15-AMC, and the tetrapeptide.

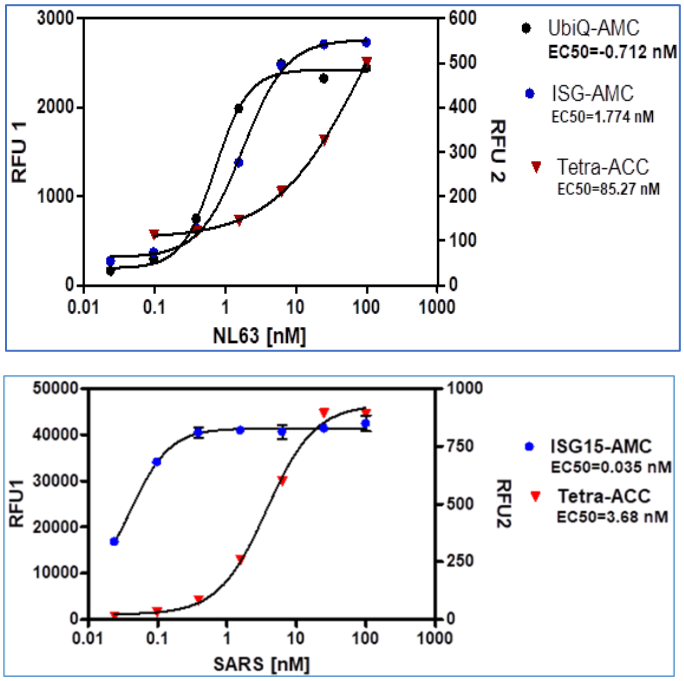


Figure 17. Activity of NL63 papain-like protease and SARS PLPro against ubiquitin and ISG15 substrates. Top. NL63 protease was serially diluted in 384-well plates and 2.5 µM Ubiquitin-AMC substrate (black circles), 166 nM ISG15-AMC (blue circles), or 10 µM tetrapeptide (red triangle) added. RFU1 correspond to the tetrapeptide and RFU2 to the other substrates. The EC_{50} values were obtained using GraphPad, nonlinear fit.

Bottom. SARS PLpro prefers ISG15-AMC over the tetrapeptide. Conversion of ubiquitin was not observed.

Result: NL63 protease can cleave substrates with the LXGG motif, with a preference for UbiQ (EC_{50} = 0.712 nM) > ISG15 (EC_{50} =1.774 nM) > tetrapeptide (EC_{50} = 85.27 nM). In contrast, PLPro from SARS-CoV-2 converts ISG15 105 x better than the tetra peptide. No appreciable cleavage of ubiquitin was observed.

7.1 Cleavage of PLpro substrates by non-viral proteases

Rationale. Human cells contain enzymes that are known to cleave substrates containing the amino acid motif LXGG. We therefore first determined if the cleavage from endogenous enzymes in cells from MRC-5 would obscure the detection of cleavage from viral enzymes.

Approach: Human lung fibroblast cells from the cell line MRC-5 (ATCC® CCL-171) were grown using standard tissue culture conditions, trypsinized and counted using a hemacytometer. Cells were lysed in Mesa Photonics lysis buffer and lysate from $0.5e^6$ cells added to 2 sets of duplicate wells of a 96-well plate containing varying concentrations of substrates (10 µM hTYR-Dap-GG-AMC, 10 µM LRGG-AMC, 100 nM Ubiquitin-AMC, 100 nM ISG15-AMC, 2.5 µM ACE2-FITC). 40 µl of 2x assay buffer was added to one set of

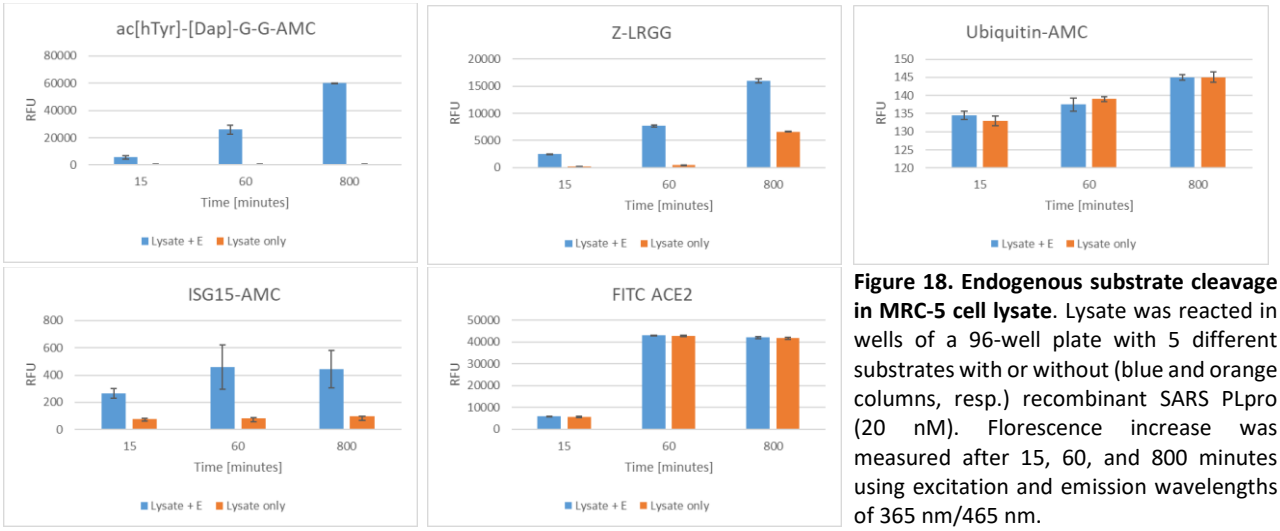


Figure 18. Endogenous substrate cleavage in MRC-5 cell lysate. Lysate was reacted in wells of a 96-well plate with 5 different substrates with or without (blue and orange columns, resp.) recombinant SARS PLpro (20 nM). Florescence increase was measured after 15, 60, and 800 minutes using excitation and emission wavelengths of 365 nm/465 nm.

wells and 40 µl PLpro enzyme (20 nM) to the other. The increase in fluorescence was monitored using a fluorescence plate reader (Tecan) after 15 minutes, 60 minutes and 800 minutes.

Result: As expected, the substrate containing unnatural amino acids (ac[Tyr]-[Dap]-G-G) was cleaved only by recombinant PLpro and not by endogenous human enzyme (Fig. 18, top left). In contrast, the substrates LRGG was slowly cleaved over time (top middle) and ubiquitin immediately (top right) by endogenous enzymes. The substrate ISG15 (bottom left) showed background fluorescence that did not increase over time and was ~ 4.5 times lower that the maximum signal generated in the presence of recombinant SARS PLpro. As expected, the substrate recognized by ACE enzymes was cleaved by endogenous ACE2 in lysate regardless of the presence or absence of SARS PLpro (bottom right).

Conclusion. The substrates containing unnatural amino acids and ISG15 are stable in lysate whereas the substrate LRGG and ubiquitin are cleaved by endogenous enzymes.

7.1.1 Substrate cleavage by viral protease from 229E coronavirus

Rationale. Identifying the cleavage of any of our reporter substrates by infected cells versus non-infected cells will provide an estimate of the test sensitivity, i.e., the number of infected cells needed.

Approach. MRC-5 cells were infected with either 15,000, 3,750 or 0 virions for 24 hours. The cells were washed, trypsinized, counted and lysed in Mesa Photonics lysis buffer. Lysate corresponding to 5,760 cells was added to duplicate wells of a 96-well plate containing varying concentrations of substrate (10 µM hTYR-Dap-GG-AMC, 10 µM LRGG-AMC, 100 nM Ubiquitin-AMC, 100 nM ISG15-AMC, 2.5 µM ACE2-FITC). The fluorescence increase was measured after 15 minutes using excitation and emission wavelengths of 365nm/465 nm for AMC and ACC or 490nm/540nm for FITC.

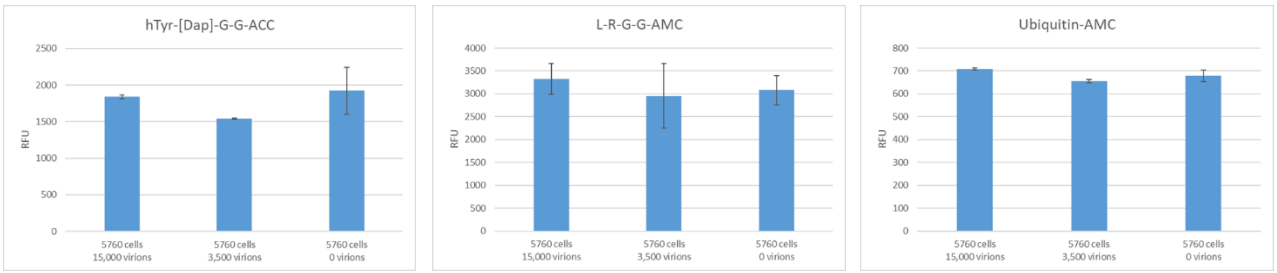


Figure 19. Substrate cleavage by papain-like protease from 229E coronavirus. Three different substrates were added to wells containing lysate derived from 5760 MRC-5 cells infected with 15,000, 3,500 or no 229E virions from the 229E coronavirus strain. The increase in fluorescence was measured after an incubation at room temperature for 15 minutes.

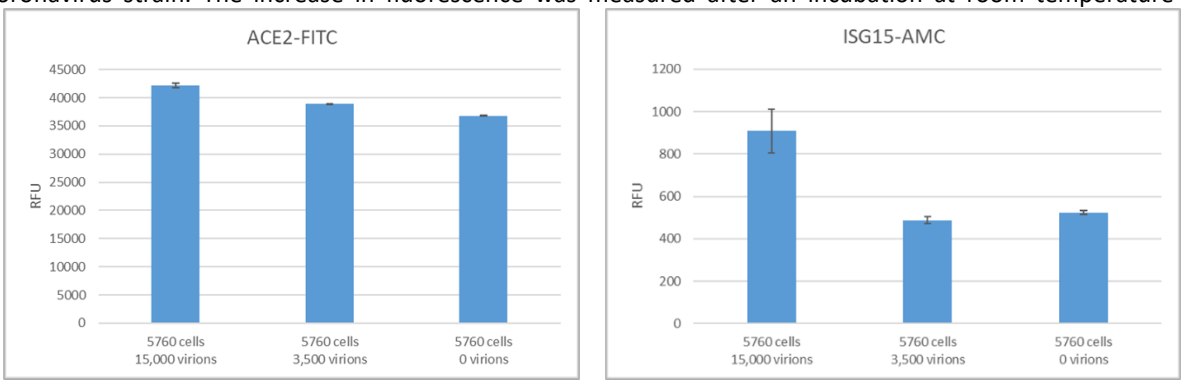


Figure 20. ACE2 and ISG15 substrate conversion in 229E infected MRC-5 lung cells. ACE2 and ISG15 substrates were added to wells containing lysate derived from 5760 MRC-5 cells infected with 15,000, 3,500 or no 229E virions from the 229E coronavirus strain. The increase in fluorescence was measured after an incubation at room temperature for 15 minutes.

The experiment above was repeated with cells that were incubated with virus for 14 hours. A 15% and 20% increase in fluorescence relative to the no virus control was obtained for substrate ISG15 in cells transfected with 15,000 or 7,500 virions (not shown).

Results. None of the 3 substrates of figure 19 are converted by 229E papain-like protease.

Interestingly, the conversion of ACE2 (Figure 20, left) was highest in lysates from cells with the highest viral load. This could be due to the fact that the ACE substrate is also recognized by caspases, which are expressed in cells about to die.

As seen in Figure 20, right ISG15-AMC cleavage was observed in lysate from cells infected with 15,000 virions, relative to lysate from cells infected with 3,500 or no virions.

Conclusion. This experiment demonstrates that the limit of detection of our assay will be well within the range expected for human samples: the tongue has around 30,000,000 cells/cm^2, and conservative estimates expect around 1% of those cells to be ACE positive, i.e., potentially infected by SARS coronavirus. This results in a target population of around 300,000 cells that are potentially infected with between 10 – 100 virions. Thus, we can expect a tongue scrape lysate to contain between 3 million to 30 million virions, which is 200 to 2000-fold higher than the 15,000 virions we were able to detect in ~6000 cells.

7.1.2 Substrate cleavage by viral protease from NL63 coronavirus

Coronavirus NL63 was transfected into rhesus monkey kidney cells LL MK2 (ATCC TCC® CCL-7) for 12 hours. Lysates from these cells were added to substrates as described in 7.1.1 and measurements taken after 15 and 60 minutes.

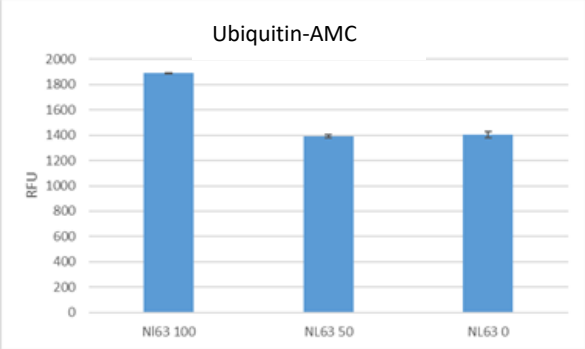


Figure 21. Conversion of ubiquitin by NL63.

Ubiquitin-AMC was added to wells containing lysate derived from ~ 24,000 LL-MK2 cells infected with 24,000, 12,000 or no NL63 virions. The increase in fluorescence was measured after an incubation at room temperature for 15 minutes.

Result. Ubiquitin cleavage was detectable in cells infected with NL63.

Conclusion. The result confirms the ubiquitin substrate preference seen with recombinant NL63 (Figure 17) and reported in the literature.[24]

7.1.3 Substrate cleavage by viral protease from OC43 coronavirus

Coronavirus OC43 was transfected into rhesus monkey kidney cells human epithelial colon cells HCT-8 (ATCC® CCL-244™) for 5 days. Lysates from the cells were added to substrates as described in 7.1.1 and measurements taken after 15 and 60 minutes.

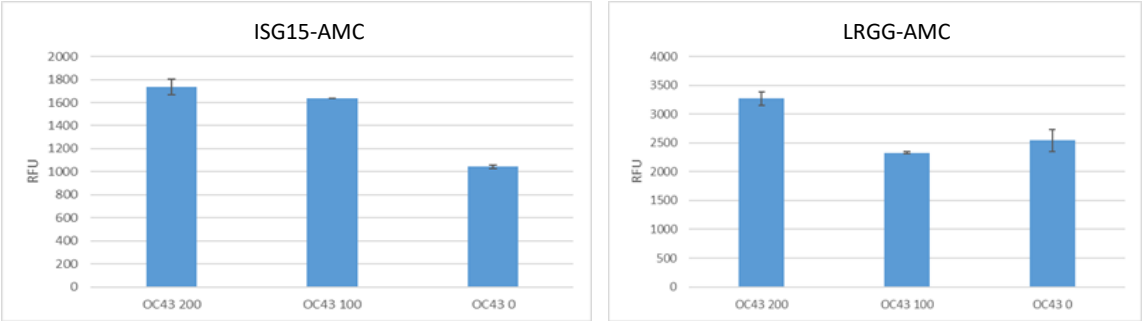


Figure 22. OC43 conversion of substrates ISG15 and LRGG. Substrates were added to wells containing lysate derived from ~ 24,000 HCT-8 cells infected with 48,000, 24,000 or no OC43 virions. The increase in fluorescence was measured after an incubation at room temperature for 15 minutes for ISG15 and 60 minutes for LRGG.

Result. ISG15 cleavage was detected after 15 minutes in lysate from cells infected with 48,000 and 24,000 OC43 virions but was on longer distinguishable from the uninfected control sample after 60 minutes. OC43 is the only coronavirus we studied that shows cleavage of LRGG after 60 minutes.

Conclusion. The kinetics of cleavage will require further study. It appears as if viral activity dominates substrate cleavage for up to ~ 15 minutes until cleavage from endogenous enzymes catches up. A control experiment that was run in parallel and contained the same amount of substrate but recombinant enzyme shows that the maximum RFU achievable from this substrate is ~ 1800, i.e., the substrate was completely converted after 15 minutes in lysate.

Phase 2 experiments will include kinetic measurements in 1-minute intervals. It is conceivable that the test could be run in less than minutes. Substituting LRGG cleavage site (which is recognized by endogenous enzymes as seen, for example, in Figure 22, right) with hTyr-Dap-GG (which we show is not cleaved by endogenous enzyme) is expected to substantially improve the test performance.

The results of our studies are summarized in Table 2.

Table 2: Comparison of substrate preferences of 7 human coronaviruses

Coronavirus	Subgroup	Ty-Dap-GG	LRGG	Ubiquitin	ISG15	Enzyme	Reference
MERS	β	-	+	++	+++	recombinant	[23,25]
SARS-1	β	++	++	++	+++	recombinant	[23,25]
SARS-2	β	+	+	++	+++	recombinant	[23,26]
OC43	β	-	+	-	++	viral, in vivo	Mesa/LANL
HKU1	β						
229E	α	-	-	-	++	viral, in vivo	Mesa/LANL
NL63	α	-	-	++	-	viral, in vivo	Mesa/LANL
NL63	α	-	-	+++	++	recombinant	Mesa

Table 2. The seven human coronavirus and their subgroups are listed. The substrate preferences of their papain-like proteases for four substrates tested is shown, where each + corresponds to approximately 1 order of magnitude. The substrate preference for HKU1 is not known and was not tested.

To our knowledge, our data is the first to demonstrate cleavage of ISG15 by OC43 and 229E. In Phase 2, these experiments will be repeated and HKU1 included and the results submitted for publication.

These results greatly reduce the uncertainty of success for our SARS test system in human samples.

8 Commercialization/Transition Path

8.1 Business Case

8.1.1 Continued need for COVID testing and widespread screening

The COVID pandemic has ravaged the world. Delayed and inaccurate testing has enabled the virus to spread through communities and across countries and continents, leaving an estimated 150 million people infected with SARS-CoV-2 and over 3 million dead.[17]

Despite the rapid development of highly efficient COVID vaccines it is not expected that COVID can be eradicated, as was the case with smallpox. Humans are not the only reservoir for coronaviruses; bats, mammals, livestock, camels are known zoonotic hosts in which the next novel coronavirus could be generated and gradually evolve in an intermediate host after mutation and recombination. It is also not expected that herd immunity can be reached globally, even if vaccines were available for all, because part of the population resists vaccination (an estimated 30% in the US).

In addition, mutants are quickly emerging from the enormous reservoir of infected people. Some of these variants can evade detection and escape natural or vaccine-derived immunity. As a result, tests and vaccines must be constantly changed to adapt to these variants and undergo renewed FDA approvals.

Clearly, continued surveillance and widespread screening will be needed to contain SARS-CoV-2, now and in the future.

8.1.2 Activity-based Diagnostics (ABDx) as a Platform for Other Current and Emerging Diseases in Humans and Livestock

Mesa Photonics platform is based on activity diagnostics which is being recognized as an emerging paradigm for disease detection and monitoring not only in humans but in livestock as well.[27] The relative ease of assembling a detection platform—once the genetic sequence of a pathogen is known—will allow for rapid deployment of a diagnostic test for future pathogens. The steps to developing an ABDx assay consist of **(a)** identifying a specific substrate through a combinatory peptide substrate screen, **(b)** recombinantly expressing the enzyme of interest, and **(c)** developing an assay and screening for specificity. Once our test system, consisting of the assay and fluorometer, have received FDA authorization, Emergency Use Authorization (EUA) for a new test could be obtained within weeks.

9 Commercialization Strategy

9.1.1 Product

Mesa Photonics primary product is an on-site test system consisting of test reagents and a portable test instrument to be used for detecting SARS coronaviruses in tongue scrape samples. The initial test will include buffers and substrates in stable and freeze-dried form, food-grade tongue scrapers, lysis buffer, sample transfer pipette, and a single sample fluorometer for which Mesa Photonics will enter an OEM agreement with Turner Designs (Figure 21). A multi-sample tester that can measure 10 samples at a time is being designed by Mesa Photonics engineers.

Figure 23. Mesa Photonics Prototype Test Kit. Tongue scrape samples will be taken with a food-grade tongue scraper **(1)** and the sample transferred with a single channel pipette **(2)** and tip **(3)** to a vial containing lysis buffer **(4).** A defined volume of the lysate is then transferred to a 0.5 ml PCR tube **(5)** that contains all reaction components in a freeze-dried, stable form. The tube is then placed in a sample holder **(6)** which in turn is inserted into the port of a single sample fluorometer **(7)** for simultaneous measurement of SARS PLpro and ACE2 activities. Measurements are taken immediately and after 15 minutes and the difference in fluorescence units calculated to derive at the diagnosis. Alternatively, the data export port on the fluorometer can be used to automatically transfer the data to an excel spreadsheet. The kit components can be assembled in a Pelican case **(8)** or in a ruggedized case that can be parachuted to remote locations.

In addition to rapid SARS diagnostics, the test components lend themselves to research applications, such as the direct detection of live virus in infected cells and drug screening applications.

Other activities related to this work:

Invited presentation: The results of our work were presented at a super symposium at CLEO on May 11, 2021. The recorded PowerPoint presentation is attached to this report.

Provisional Patent Application. A broad provisional patent was filed on 07-20-2020 to cover the ABDx approach for screening of SARS and other current and emerging pathogens. The application is being converted to a utility application by our patent lawyers at the Peacock Law firm in Albuquerque, NM.

9.1.2 Customer Base

Our technology is of particular interest for decentralized testing in non-POC settings and for use in low resource settings where centralized testing capabilities are scarce. The expected end-users of non-POC tests in a CLIA-waived environment are businesses, borders, schools, colleges, airports etc. Mesa Photonics will work with its partner Spectrum Solutions for bulk manufacturing and distribution through Spectrum Solutions customer network that aggressively markets its products internationally.

We also expect to commercialize to laboratories, hospitals, diagnostic centers, and clinics. Although centralized testing sites have made large investments in RT-PCR instruments, we believe that the competitive features of our test will spurn interest for higher throughput testing in plates, e.g., 96-well plates, in plate-based fluorescence plate readers that are standard laboratory equipment. For those applications, we would sell our reagents in bulk.

In addition, our technology is an excellent fit for non-governmental organizations (NGO's) that purchase and distribute diagnostics for the developing world. The World Health Organization (WHO) has defined a set of criteria for limited resource environments: the diagnostic test system must run on battery power,

require no refrigeration and be simple to operate. Our product will meet these specifications and perform high quality tests for USD 10 per test in a USD 3,000 battery-operated fluorometer.

Pharmaceutical companies that are engaged in developing vaccines and/or therapeutic antibodies are another source of potential customers. The expected annual COVID-19 vaccines must establish efficacy in clinical trials involving several hundred subjects. Our test system would be an ideal companion diagnostics tool for these trials where subjects take the test system to the home and send daily results through an app to the clinical trial center. A 30-day monitoring period for each subject in a 300-member cohort equipped with one of our test systems would produce ~ USD 180,000 in sales for Mesa Photonics for each vaccine or therapeutic undergoing clinical testing in the USA. Indeed, AstraZeneca has expressed interest in our test for this companion diagnostics application, once the system is approved for a CLIA waived setting.

A future application of our on-site test system is for diagnostics in livestock, where the increasing prevalence of food borne and zoonotic diseases has stimulated the demand for advanced animal diagnostic solutions. An important advantage of targeting the livestock industry is that FDA pre-market approval or submission of a 510(k) for the device is not required.

9.1.3 Market Analysis

The Global COVID-19 diagnostics market is estimated to account for USD 84.4 billion in 2020 and is expected to expand at a compound annual growth rate (CAGR) of 3.1% to reach an estimated value of 104.7 billion by 2027.[28] The high numbers of new infections with wildtype and variants are driving the market. The market is then expected to stabilize and reach $4.73 billion in 2023 at a CAGR of 10.36%. Although a gradual decline in the number of cases is expected, testing processes are expected to witness growth owing to the fact that COVID-19 is anticipated to become part of routine testing for patients with flu-like symptoms.

The global COVID-19 rapid test kits market is expected to reach $3.52 billion by the end of 2020 and expected to be $4.22 billion by 2021 at a compound annual growth rate (CAGR) of 19.7%.[29]

We anticipate that initially we could capture around 1% of the global rapid test kit market of approximately 40 million and then ramp up to sales revenues of 400 million/year based on the anticipated selling price of USD 10/test and USD 3000/fluorometer.

If the test is successful, Mesa Photonics is open to licensing or sublicensing agreements, for example, with pharmaceutical or medical companies.

9.2 Transition Plan

The Phase 2 work will consist of the following efforts:

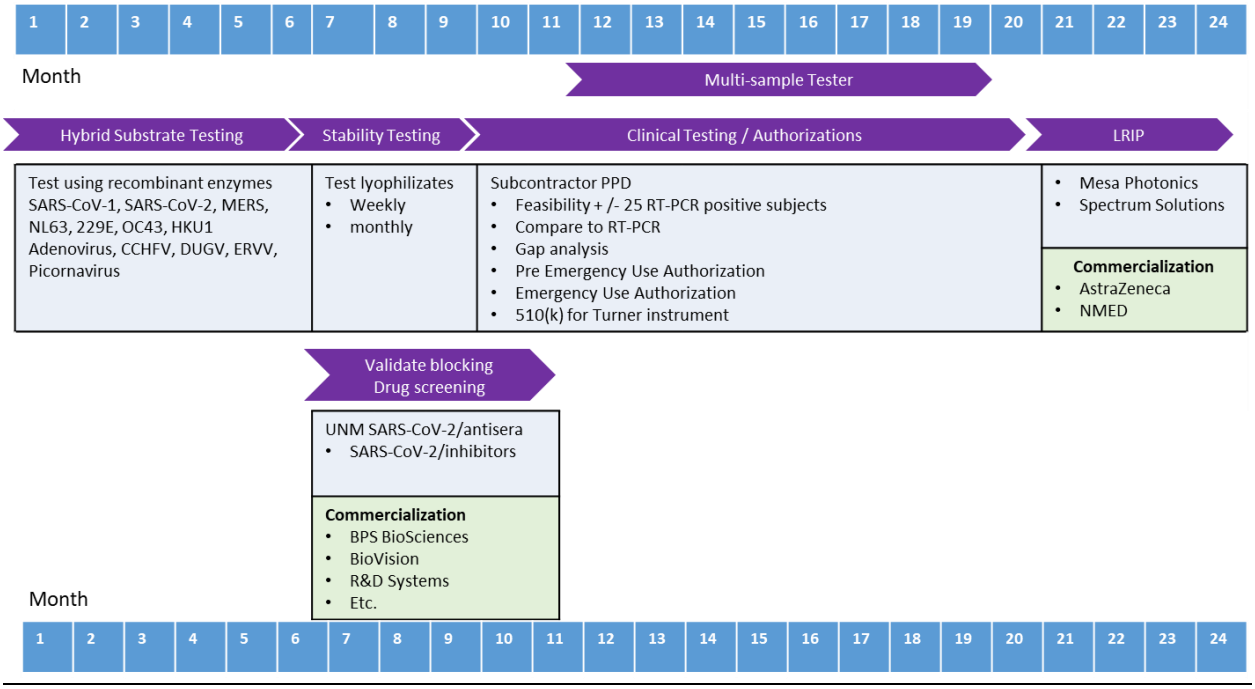
1. Generate and test a substrate consisting of ISG15 in which the terminal LRGG is replaced with Tyr-Dap-G-G-AMC to combine sensitivity and selectivity. Substrates will be synthesized by Genscript (Piscataway, NJ). The substrate will be tested using recombinant enzyme from all 7 coronaviruses and from 5 other viruses known to express proteases that cleave ISG15. Five of these enzymes are commercially available; 7 will be recombinantly expressed by Creative Enzymes (Shirley, NY).
2. Once the optimal substrate configuration and concentration are established, stability testing of the reagents will be started. The reaction buffer and substrates will be combined and lyophilized in a Labconco freeze-drier available at the Santa Fe Business Incubator (SFBI). Testing will be performed in weekly and then monthly intervals for 4 months.
3. In parallel with stability testing, we will validate our test reagents for determining the blocking activity of anti-SARS antibodies and as a cell-based drug screening platform. These tests will be performed at

the University of New Mexico (UNM) by our collaborator using commercially available human antisera and known drugs. The data will be incorporated into product information sheets for advertising purposes by companies such as BPS Biosciences, BioVision, R&D Systems etc. These companies have a broad customer base in the SARS reagent business through which our products will be distributed.

4. Clinical testing will be performed by our subcontractor PPD (Wilmington, NC). This will include feasibility testing in 25 subjects with and without SARS-CoV-2 as determined by RT-PCR. The outcome will inform us on the accuracy of our test in clinical samples. Gap analysis will be performed in preparation for obtaining Emergency Use Authorization (EUA) for a test with a non-approved platformed and an application for EUA submitted to the FDA. Lastly, PPD will apply on our behalf for 510(k) clearance for the Turner instrument. If successful this clearance will serve as the predicate for obtaining 510(k) clearance for our multi-sample tester at the end of Phase II.
5. A multi-sample prototype will be developed based on our Phase I engineering designs.
6. At the end of year 2, Mesa Photonics is ready for low-rate initial production of test kits with Turner Design as our OEM manufacturer. Potential customers of our test system as a companion diagnostics test include AstraZeneca and other pharmaceutical companies who develop vaccines and/or therapeutic antibodies. We will also request support from the New Mexico Economic Development Department (NMEDD). We have successfully competed for $ 100K grants from NMEDD in the past and plan to do so again for further testing of our system, e.g., at Christus St. Vincent Hospital in Santa Fe, NM.

Higher volume production will be possible through our manufacturing partner Spectrum Solutions who also offer chemical formulations, custom packaging, specialized kitting, and direct-to-user product fulfillment. Spectrum Solutions global footprint will provide access to foreign markets.

The time line for transitioning our product is summarized below.



During our Phase I efforts, we have identified several important partners who will support us in the further development of the test system. These include

- **Turner Designs,** OEM agreement for single sample tester
- **Los Alamos National Laboratory,** Biosecurity and Public Health, B10 Division
- **University of New Mexico**, Steven B. Bradfute Center for Global Health; Division of Infectious Diseases; Department of Internal Medicine; Albuquerque, NM
- **PPD,** Clinical Research Organization for Clinical Trials, Wilmington, NC
- **AstraZeneca**, David Hayes, Emerging Innovations Unit, Waltham, MA
- **Spectrum Solutions**, Bill Phillips
- **Michael Anderson**, Senior Advisor to HHS at Children's National Hospital, Washington, District of Columbia, United States

The cost for transitioning our product is shown in the tentative budget below.

Total Labor	**195,818**
Consultants	8,308.00
Sub-contractor	300,000.00
Rent	14,400.00
Conferences	5,000.00
Materials & Supplies	100,000.00
Total Direct Costs	**600,072**
Overhead	113,065.00
Indirect Costs	713,138.00
G&A	68,389.00
Total Costs	1,494,668.00
Profit	10,462.00
Total costs	**1,599,293**

10 Literature

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