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NowAware: Rapid Enzyme Activity Detection (*REaD*) of SARS-CoV-2

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[1]Bambu Vault

prai : Prakash Rai

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Coronavirus Method Development Community

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ABSTRACT

This protocol describes the step-by-step procedure to perform the Rapid Enzyme Activity Detection (REaD) test for SARS-CoV-2 detection from NowAware, Inc.

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KEYWORDS

Microplate Reader, Enzyme-Substrate, Kinetics, Fluorescence Assay, Mobile Reader (fluorescence emission), Biochemical Reaction, SARS-CoV-2, Detection, Diagnostic, Treatment Monitoring

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GUIDELINES

It is the responsibility of the person performing the test to follow this protocol as described. The authors do not take any responsibility for any errors or misinterpretations that may occur if the protocol is not strictly adhered to as described.

MATERIALS

NAME	CATALOG #	VENDOR
Bovine Serum Albumin (BSA), Fraction V, Protease Free	A-420	Gold Biotechnology
DMSO, ACS Grade	D-360	Gold Biotechnology
DTT (Dithiothreitol) (> 99% pure) Protease free	DTT	Gold Biotechnology
HEPES, Free Acid	H-400	Gold Biotechnology

NAME	CATALOG #	VENDOR
Sodium chloride	S7653	Sigma Aldrich
PLpro Enzyme from SARS-CoV-2	E-611-050	R&D Systems
Z-RLRGG-AMC	4027158.01	bachem

MATERIALS TEXT

Consumables like pipetes, tips, 96-well microplates etc are not included. These are standard in any biochemistry/biology/biotechnology lab.

F Nano+
Dual-mode Micro-plate Reader

Infinite **TEC006430I**

Filters that can excite at 395nm and read emission at 440nm are needed.

EQUIPMENT

NAME	CATALOG #	VENDOR
F Nano+	TEC006430I	Tecan

SAFETY WARNINGS

1. Handle all biochemicals as per the CDC's Biosaftely Level 2 guidelines and protocols

2. For sample collection - patients should be strictly advised not to eat, drink, chew gum, brush their teeth or tongue, or use any mouthwash at least 30 minutes before specimen collection.

3. Negative results do not rule out SARS-CoV-2 infection, particularly for patients who have been in contact with known infected persons or in areas with high prevalence of active infection. Follow -up testing with a molecular diagnostic test may be necessary to rule out infection in these individuals. Results from NowAware COVID-19 test should not be used as the sole basis to diagnose or exclude SARS-CoV-2 infection. False positive results may occur due to cross -reacting enzymes from the human or infections, such as other coronaviruses, or from other causes. Samples with positive results should be confirmed with alternative testing method(s) and clinical findings before a diagnostic determination is made.

BEFORE STARTING

Assure all the testing components for the REaD test are located at the testing lab and are consistent with the specifications and storage guidelines setout in this protocol.

Specimen Collection and Preparation

1 Assure the following testing components for pREaD test are located at the testing lab and are consistent with the following specifications and storage guidelines:

Sample Container – This container needs to house the Buffer, Substrate, and Sample Collection tool while also allowing the administrator of the test to see inside the container to ensure a proper sample has been taken. As there is a strong likelihood that these containers will need to be transported from one location to another, ensuring that they have a water-tight seal and are reasonably durable such that they are not damaged or leak in transit.

The Dimensions for the container should be as follows:
•**Length**: ~1.75" to 3"
•**Outer Diameter:** 5/8th of an inch
•**Inner Diameter:** 3/8th of an inch

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•**Suitable Materials:** Polypropylene, High Impact Polystyrene (HIPS)

Lysis Buffer – This buffer is used to lyse the cells and viruses in the clinical sample allowing access to the enzyme. The sample transport medium also serves as the medium in which the enzyme substrate reaction occurs. The sample transport medium also serves as the medium in which the enzyme substrate reaction occurs. Lysis buffer completely deactivates the virus by destroying its lipid envelope and is no longer hazardous. The Lysis Buffer is stable at room temperature for two weeks and at 4°C for over six weeks.

Precise Make-Up of Lysis Buffer

•pH 7.5
•50 millimolar(mM) HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) ,
•150mM NaCl,
•0.1 mg/ml Bovine Serum Albumin (BSA)
•2.5mM Dithiothreitol (DTT) – reducing agent
•0.5% Triton X-100 in distilled water

Peptide Stock – Fresh peptide stock can be made by taking aliquot of peptide powder and dissolving it in the dissolution buffer before testing begins. The substrate is a 5-amino acid peptide (RLRGG) attached to a chromophore 7-Amino-4-methylcoumarin (AMC) and is referred to as RLRGG-AMC. The substrate is provided as a powder and can be readily dissolved using buffer described below. Peptide Stock is stable at room temperature and at 4°C for over a month (45-day time point has been tested).

Precise Make-Up of Peptide Stock

•50 millimolar(mM) HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid),
•10% dimethyl sulfoxide (DMSO)
•Peptide stock solution is 5 mg/ml and 1ml of this stock solution is sufficient for an entire 96-well plate

•**Isohelix Buccal Swab** – Tongue sample collection device is used to scrape infected cells off the tongue and transfer into the collection tube.

Technical Specifications of Isohelix Buccal Swab:

•**Optimal Cellular Material pickup**: 100-125 mg
•**Optimal Volume of sample picked up:**0.1 ml
•**Overall Length**: approximately 6-6.5”
•**Length of Sample Collection Tool intended to fit into sample collection tube**:
1.5” (i.e the collection tool should have a ridge to snap off the shaft at ~1.6”)
•**Thickness of Shaft** – $1/8^{th}$ of an inch
•**Max Thickness of Brush or Head (piece that brushes the surface of the tongue):** ~0.25”

2 Consider any materials of human origin as infectious and handle using standard biosafety procedures. Patients should be advised not to eat, drink, chew gum, brush, or use any mouthwash at least 30 minutes before specimen collection.

3 Using an Isohelix buccal swab, gently rub one side of the swab across one half of the top of the tongue about 5 times, repeat again on the other side of the top of the tongue 5-times, flip the swab and repeat this procedure.

4 Place swab with tongue sample carefully into the container containing lysis buffer and snap shaft apart at indentation/ridge so that it fits into the tube. Close lid tightly. Gently shake the tube, Remove swab after 5-minutes and close lid again.

5 Container containing sample is left at room temperature for 30 minutes to complete lysis of cells. Note this step can occur while the samples are being transported to a testing lab from the sample collection center.

Sample Shipping and Storage

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6 Samples can be stored at 2-8°C for up to 72 hours after collection. Do not freeze the specimen. Do not expose it to high temperatures (>75 °F).

7 Samples will then be sent to be processed using either the plate reader (pREaD) or mREaD/iREaD. When specimens are transported, they must be kept at the 2-8°C at all times. These temperatures can be maintained by having the tests transported with cold packs. Specimens must be packaged, shipped, and transported according to the current edition of the International Air Transport Association (IATA) Dangerous Goods Regulations.

Plate Reader (pREaD)

8 All the measurement and analytical functions are performed by a microwell plate reader instrument, such as a BioTek Synergy Neo2 multi-mode reader. Create a plate layout in the plate reader to map sample locations.

9 Take out a new 96-well black plate (Corning Falcon Black plates) with clear bottom.

10 Populate control wells in plate with four prescribed controls – two internal controls and two external controls:

Our internal controls included with every multi-well plate are as follows:
1. The substrate alone in lysis buffer with no enzyme added (“Negative Control”).
2. The equivalent amount of the substrate with 10 nM of purified PLpro enzyme (“Positive Control”).

Our External control samples are tested in every multi-well plate include:
1. A previously tested known positive (by PCR) clinical sample with equivalent amount of substrate added.
2. A previsously tested known negative (by PCR) clinical sample with the same amount of substrate added.

11 Open samples one at a time and gently homogenize the sample by pippetting up and down (without creating bubbles).

12 From the mixed sample, pipette in 90µL from the bottom of vial. Pipette out 90 µL into an empty well according to the plate layout. One 96-well plate can have as many as 92 tests.

13 Pipette 10µL of the substrate from the peptide stock (5mg/ml stock) into each well and gently mix with the sample without creating bubbles.

14 Using a set predefined protocol for the plate reader- the 20 minute kinetics and fluorescence end point(395nm excitiation with 440nm emission) after 20 minutes has to be recorded. with mean Velocity calculated automatically by the plate reader software at the end of 20-minute kinetic run

15 Load and run plate through the plate reader, and at the end of the run, take it out and cover it.

16 Verify on the plate reader
•The negative control should have a fluorescence intensity under 200 RFU
•The positive control should have a fluorescence intensity above 600 RFU
•The negative clinical sample control should have a fluorescence intensity under 300 RFU
•The positive clinical sample control should have a fluorescence intensity over 400 RFU

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•Positive and Negative Controls should be tested to ensure the proper performance of the assay, particularly under the following circumstances.

- A new operator uses the kit.
- A new lot of test kits is used.
- A new shipment of kits is used.
- The temperature used during the storage of the kit falls outside of 4-25 °C.
- The temperature of the test area falls outside of 4-25 °C.
- To verify a higher than expected frequency of positive or negative results.
- To investigate the cause of repeated invalid results; or
- A new test environment or equipment is used.

Plate Reader (pREaD) Data Analysis

17 Download results from the plate reader using plate reader software or LIMS.

18 Using Microsoft Excel or equivalent software, open the results file from the plate reader and calculate average relative fluorescence units (RFU) for all patient samples and controls (if running in duplicates or triplicates etc).

19 Calculate RFU ratio by dividing average RFU for sample with that of the negative (no enzyme, substrate only) control.

20 Based on ratio of RFU for sample with respect to the negative control, interpret patient sample results as shown below- positive and negative for SARS-CoV-2.

Interpretation of Results

•If average sample RFU is > 500 RFU and ratio of sample RFU/negative control RFU is > 1.2 sample is considered positive

•If average sample RFU is < 400 RFU and ratio of sample RFU/negative control RFU is < 1.15, sample is considered negative.

•If average sample RFU and ratio of sample RFU/negative control RFU lies in between these values, please re-run the sample to confirm status of the result. If result is repeated, sample is considered indeterminate. Get another sample from the patient and repeat the test.

•Always check the RFU values of the negative control, positive control and clinical sample for reference when interpreting results.

21 Based on average of mean Velcoity, interpret the patient sample results as follows

- If Mean V is <1 RFU/min patient is considered negative for SARS-CoV-2
- If Mean V is >1 RFU/min patient is considered positive for SARS-CoV-2.

If either result from step 19 and 20 are not in agreement, repeat the test with the same sample again. If the discordance still persists, collect fresh sample from the patient and repeat the test.

Negative results do not rule out SARS-CoV-2 infection, particularly for patients who have been in contact with known infected persons or in areas with high prevalence of active infection. Follow -up testing with a molecular diagnostic test may be necessary to rule out infection in these individuals.

Results from NowAware COVID-19 test should not be used as the sole basis to diagnose or exclude SARS-CoV-2 infection. False positive results may occur due to cross -reacting enzymes from the human or infections, such as other coronaviruses, or from other causes

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Samples with positive results should be confirmed with alternative testing method(s) and clinical findings before a diagnostic determination is made.

Mobile Read (mREaD)

22 The sample collection and storage/shipping steps are identical. mREaD uses a custom device to read the fluorescence emission. The mREaD device comprises a holder for a sample cuvette, an LED emitting at 365 nm that optically excites components in the sample cuvette, a sensor that detects fluorescence light from the cuvette, electronics to amplify the sensor signal and to convert the analog signal into a digital output.The device makes use of additional software that provides control of light intensity, data access, and writing data to or reading from EEPROM.

Generally, the use of the mREaD reader begins with calibration of the reader with a calibration sample that represents the response of the minimum fluorescence required to assess a clinical sample as a positive result.This calibration is recorded in EEPROM and used as a reference value to determine if the sample is considered to be a positive test for virus.

Data communication can be performed with a USB connection to a computer or cellular phone.Alternatively, a Bluetooth connection can be used for both the device controls and sample data.

23 180 µL of patient sample is combined with 20 µL of 5 mg/mL substrate in 50 mM pH 7.5 HEPES buffer with 10% DMSO inside a Brandtech ultra-micro cuvette and gently shaken to ensure components were well mixed.

24 Exactly, 20 minutes after substrate addition, the cuvette containing the sample and the substrate is measured in the mREaD device.

25 The fluorescence values are compared to a standard that represents the minimum fluorescence shown in a COVID-19 positive sample.If the sample being tested shows equivalent or greater fluorescence than the standard, it is a positive result; if the fluorescence is lower than the standard, it is a negative result.

Biowaste Handling

26 Sample/Waste Handling: Lysis buffer contains Triton X-100, which is a non-ionic surfactant, that destroys lipid membranes and completely deactivates the virus by destroying its lipid envelope, making the sample no longer hazardous to anyone working with it.

•CDC has approved this work under BSL-2 certification.

•All waste generated during the **p*REaD or mREaD/iREaD*** test can be put into a 10% bleach solution, which can be left for 30 minutes and discarded as any biowaste generated under BSL-2 certification.

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