Title
Development and Clinical Validation of EFIRM Detection of Functional Neutralizing-Antibodies Against SARS-CoV-2 in Saliva

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Development and Clinical Validation of EFIRM Detection of Functional Neutralizing-Antibodies Against SARS-CoV-2 in Saliva

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Oral Biology

by

Aida Mohammadi

2023

ABSTRACT OF THE THESIS

Development and Clinical Validation of EFIRM Detection

of Functional Neutralizing-Antibodies Against SARS-CoV-2 in Saliva

by

Aida Mohammadi

Master of Science in Oral Biology

University of California, Los Angeles, 2023

Professor Yong Kim, Chair

Objectives: Despite extensive research on the blood neutralizing antibodies (NAbs) to SARS-CoV-2, relatively little is known about the level of saliva NAbs and how it relates to systemic NAb levels. Current emergency use authorization (EUA) serology assays only include measuring NAbs in plasma or serum and there is no test for measuring NAbs in saliva. The aim of this study was to develop a saliva-based assay for measuring COVID-19 NAbs and compare the level of NAbs in saliva and plasma.

Methods: The EFIRM (Electric Field Induced Release and Measurement) technology, is a novel platform that can quantify target molecules in both blood and saliva. The EFIRM NAb assay was developed using human angiotensin-converting enzyme 2 (hACE2) protein immobilized onto a gold electrode. The protein-protein interaction between the virus receptor binding domain (RBD) and hACE2 is disrupted by NAbs against SARS-CoV-2 RBD, if present in a clinical sample. Paired plasma and saliva samples collected from COVID-recovered or

vaccinated patients were assayed for EFIRM NAb. Saliva and plasma samples collected prior to 2019 from a healthy cohort were used to determine the clinical specificity and cutoff.

Results: The EFIRM saliva NAb assay detected NAbs in saliva with a limit of detection (LOD) of 31.6 U/mL and differentiated between COVID-19-recovered or vaccinated patients (n = 31) and healthy individuals (n = 60) with an area under the curve (AUC) of 0.923 (95% CI: 0.869 to 0.976), a sensitivity of 87.10%, and a specificity of 86.67%. Comparing the level of neutralizing antibodies in paired saliva and plasma, a significant correlation was seen between neutralizing titers ($r = 0.75$, $p < 0.0001$).

Conclusion: A quantitative, non-invasive electrochemical saliva-based assay with sufficient sensitivity and specificity was developed to measure SARS-CoV-2 functional neutralizing antibodies. Our novel EFIRM NAb saliva test represents a significant technological advancement to address the unmet clinical needs for large-scale surveillance in the pandemic world and beyond with great potential future applicability.

The thesis of Aida Mohammadi is approved.

Renate Lux

Fang Wei

David T.W. Wong

Yong Kim, Committee Chair

University of California, Los Angeles

2023

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INTRODUCTION

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), a positive-sense single-stranded RNA virus, was initially isolated in December 2019 from a cluster of acute respiratory illness cases in Wuhan, China (1). During the initial two years of the pandemic, coronavirus disease 2019 (COVID-19) emerged as the third most prominent cause of mortality in the United States, only ranking behind heart disease and cancer (2). As of May 27, 2023, 6,169,122 COVID-19-related hospitalizations and 1,130,593 deaths have been reported in the United States (3). The presentation of COVID-19 demonstrates notable variability in its severity (4). The hallmark symptoms of COVID-19 include fever, cough, and shortness of breath, which can rapidly progress to respiratory and cardiac failure, often requiring the use of mechanical ventilation (5). Individuals who are elderly, immunocompromised, and those with underlying metabolic, pulmonary, and cardiac conditions face a heightened risk of mortality from COVID-19 (6). The majority of human infections occur through respiratory droplet exposure, and local and global spread is aided by asymptomatic or pauci-symptomatic individuals engaging in community transmission (7).

SARS-CoV-2 possesses various structural proteins, namely spike (S), envelope (E), membrane (M), and nucleocapsid (N). The spike protein (S) plays a crucial role as it contains a receptor-binding domain (RBD) responsible for recognizing and binding to the cell surface receptor (8). Angiotensin-converting enzyme 2 (ACE2), which is widely expressed by a variety of human cells, serves as the primary receptor for SARS-CoV-2 (9). The complex formed between the virus RBD and human ACE2 (hACE2) is responsible for the virus entry into host cells, and inhibiting the formation of this complex may prevent infection and reduce disease severity (10).

The clinical course of SARS-CoV-2 infection involves an acute phase characterized by detectable viral RNA and antigen in clinical samples, followed by a convalescent phase where antibodies are present in both saliva and serum. Consequently, simultaneous analysis of these diverse biomarkers in clinical samples throughout the disease progression holds the potential to provide more precise results for disease monitoring and management (11). Despite the significant contributions of molecular detection techniques like the polymerase chain reaction (PCR) and next-generation sequencing in the early diagnosis and monitoring of genetic variations of the virus, there is an urgent need for a reliable and versatile antibody test. Such a test is essential for retrospective contact tracing, assessing the rate of asymptomatic infections, accurately determining the case fatality rate, evaluating herd immunity and protective immune responses in recovered individuals and vaccine recipients, as well as identifying the natural reservoir and potential intermediate host(s) of the virus (12).

Antibodies have a crucial role in the adaptive immune response and are considered one of the most significant factors associated with protection against infectious diseases (13). Antibodies that recognize pathogens can be divided into two categories: neutralizing antibodies (NAbs) and non-neutralizing antibodies (non-NAbs). The distinction between these two types is typically based on their ability to block cell invasion or inhibit membrane fusion after binding to a specific pathogen. NAbs have the capability to prevent cell invasion or inhibit membrane fusion, whereas non-NAbs do not possess these blocking properties. Generally, NAbs are effective in neutralizing pathogens, reducing pathogen levels, and providing protection against infection to tissues or cells. In contrast, non-NAbs typically lack detectable neutralization activity (14).

NAbs in response to natural SARS-CoV-2 infection can typically be detected in the blood within several days following the initial infection, which is similar to the timeframe for the production of IgG binding antibodies (15). While individuals infected with SARS-CoV-2

may have detectable antibodies for several months after seroconversion, studies have indicated that the temporal persistence of neutralizing antibodies tends to decrease over time. However, there is currently no definitive evidence regarding the total duration of these antibodies (16-21). Breakthrough cases or individuals who have been both infected and vaccinated have demonstrated heightened potency, broader coverage, and longer-lasting serum-neutralizing activity compared to those who received only two doses of the COVID-19 vaccine or were solely infected by SARS-CoV-2 in 2020 (22).

Both natural infection and vaccination against SARS-CoV-2 have been demonstrated to provide a certain level of immunity, offering protection against reinfection, and reducing the likelihood of severe outcomes. Studies have indicated that seropositive recovered subjects have an estimated 89% protection against reinfection. Additionally, vaccine efficacies ranging from 50% to 95% have been reported. However, the duration of this protective immunity remains uncertain, as the initial immune response tends to diminish over time. Furthermore, there is ongoing transmission of new viral variants that may evade both vaccine-induced and natural immune responses (23).

A correlate of protection (CoP) is an immunological marker that is linked to protection against an infectious agent after infection or vaccination. Certain CoPs can be categorized as mechanistic, meaning they directly contribute to disease protection. In contrast, other CoPs are non-mechanistic or surrogate, which means they may not directly contribute to protection but can be used as a substitute for the true indicator of protection (24). Most of the CoPs are humoral and are utilized as surrogate indicators. This is because antibodies are relatively easier to detect in clinical laboratory settings compared to components of cellular immunity (25). Gaining a clear understanding of a CoP for SARS-CoV-2 is of utmost importance as it enables us to enhance our comprehension regarding the degree and duration of protection against infection for both individuals and populations (24).

Multiple studies have demonstrated a correlation between elevated levels of neutralizing antibodies and the immune protection against symptomatic SARS-CoV-2 infection during short term follow-up after vaccination. In addition, three of these studies estimated the level of protection linked to specific antibody levels, aiming to assess the connection between neutralizing antibody levels and the efficacy of the vaccine (23, 26, 27). Khoury *et al.* (23) estimated that the level of neutralizing antibodies required for 50% protection against COVID-19 was around 20% of the average titer observed in individuals during the convalescent phase (equivalent to 54 IU/mL). Feng *et al.* (26) and Gilbert *et al.* (27) indicated varying 70% protective thresholds ranging from 4 to 33 IU/mL. These variations in thresholds were attributed to the different assays employed, suggesting that assay differences might play a role in the observed discrepancies. While the studies mentioned reported specific threshold antibody levels associated with 50% or 70% protection, they all found that the level of protection changes gradually in relation to the neutralization titer. Consequently, there is no strict threshold below which individuals are unprotected or above which complete protection is guaranteed (23, 26-28).

Standard serology tests for SARS-CoV-2, which detect binding antibodies such as IgG and total antibodies, cannot distinguish between general binding antibodies and neutralizing antibodies (29). Therefore, NAb assays are the only reliable method for assessing the true protective immunity of antibodies (30). Research laboratories and pharmaceutical companies are in a competitive race to develop NAb tests that possess sufficient sensitivity and specificity to detect COVID-19 infection. There are three types of neutralizing antibody tests that are being pursued.

The current gold standard is the conventional virus neutralization test (cVNT) known as Plaque Reducing Neutralization Test (PRNT), which detects NAbs present in a patient's blood. The cVNT necessitates handling live SARS-CoV-2 in a specialized biosafety level 3

(BSL3) containment facility and is a laborious and time-consuming process, typically taking 2 to 4 days to complete. The mechanism of cVNT is shown in Figure 1. NAbs against SARS-CoV-2 block the interaction between the spike protein of the virus and the hACE2 receptor proteins present on the surface of host cells. On the other hand, the pseudovirus-based virus neutralization test (pVNT) can be conducted in a BSL2 laboratory but still requires the utilization of live viruses and cells.

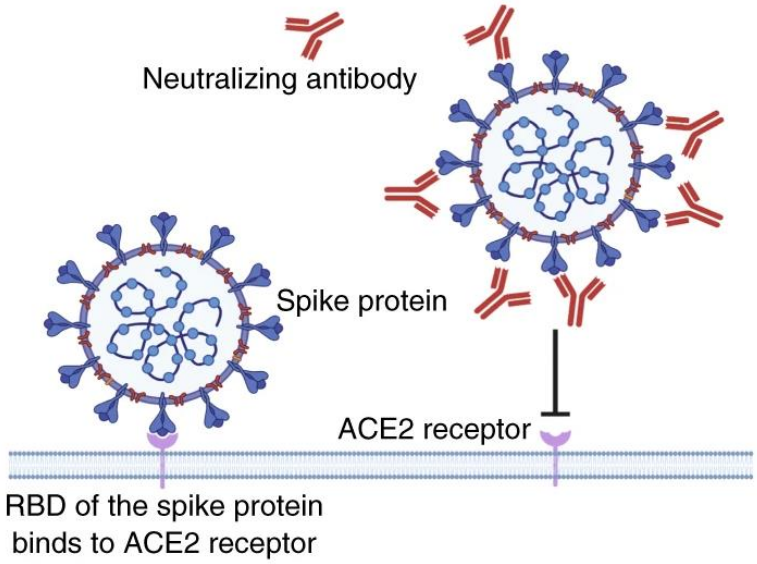

Figure 1: The mechanism of cVNT (31)

cPass surrogate virus neutralization test (sVNT) is a newly structured FDA approved assay that can detect total NAbs in plasma or serum within 1-2 hours in a BSL2 laboratory without the use of any live virus or cells. The cPass assay utilizes purified RBD from the S protein and the host cell receptor ACE2 to recreate the virus-host interaction within an ELISA plate well (Figure 2). This interaction between RBD and ACE2 can be blocked by specific NAbs present in the sera of patients, just like in the cVNT or pVNT. The cPass Neutralization Antibody Detection Kit results have shown 95.7% positive percent agreement (PPA) and 97.8% negative percent agreement (NPA) with the gold standard PRNT in clinical study (15, 29, 31-37). It is important to note that all neutralizing antibody assays exclusively detect NAbs

in plasma and serum and are not compatible with saliva samples. Due to lower antibody levels in saliva compared to plasma, the measurement of antibodies in saliva necessitates a more sensitive assay.

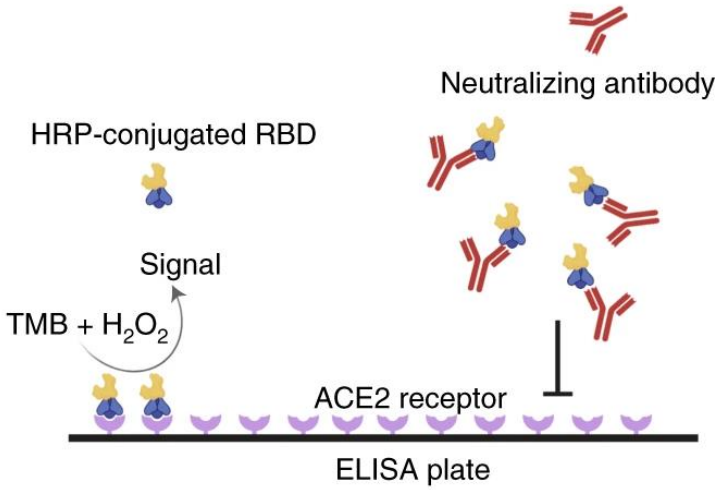

Figure 2: The mechanism of sVNT (31)

Electric Field Induced Release and Measurement (EFIRM) is a novel platform that enables the quantitation of target molecules in both blood and saliva samples. This technology operates by immobilizing capture moieties on the surface of an electrode structure, facilitating the capture of target analytes. The quantification of the target analyte is achieved through electrochemical measurements of the oxidation-reduction reaction between a hydrogen peroxide and a tetramethylbenzidine (TMB) substrate, along with the involvement of a peroxidase enzyme in a completed assay sandwich. The total assay time is less than 1.5 hours and requires only a small sample volume (less than 50 µL). The assay is conducted on electrodes that are packaged in a traditional 96-well microtiter plate format (38).

Saliva is an oral fluid that can be easily and non-invasively collected. Proteomic studies have revealed that the immunoglobulin profile in saliva closely resembles that of plasma (39). Previous studies have shown a strong correlation between levels of COVID-19 antibodies in

serum and saliva (40, 41). Isho *et al.* (40) investigated whether the levels of antibodies to the spike and RBD of SARS-CoV-2 in saliva correlate with those in serum and revealed a significant positive correlation between saliva and serum for each antigen-antibody combination. Correlations for anti-RBD and anti-spike IgG ($\rho = 0.71$, $\rho = 0.54$), and anti-RBD and anti-spike IgM ($\rho = 0.65$, $\rho = 0.58$) were stronger than those for the levels of serum and saliva anti-RBD and anti-spike IgA ($\rho = 0.39$ and $\rho = 0.54$, respectively). They suggested that saliva can be a reliable alternative for antibody testing, especially for the measurement of anti-spike IgM and anti-RBD IgG. Similarly, Sano *et al.* (41) found a positive correlation between the levels of anti-spike IgG antibodies in serum and saliva ($r = 0.9$) , while the levels of anti-spike secretory IgA antibodies in saliva did not show a strong correlation with serum IgG levels ($r = 0.57$). The findings of these studies demonstrate that detecting SARS-CoV-2-specific antibodies in saliva holds promise as a surrogate for serological testing, with salivary IgG reflecting blood-derived transudate and salivary IgA potentially indicating a local mucosal response to infection.

Despite extensive research on the blood NAbs to SARS-CoV-2, relatively little is known about the level of saliva NAbs and how it relates to systemic NAb levels. Current emergency use authorization (EUA) serology assays only include measuring NAbs in plasma or serum and there is no test for measuring NAbs in saliva. The high precision and sensitivity of the EFIRM platform enabled us to design an EFIRM NAb assay that can detect NAbs in saliva samples by successfully replicating the virus-host interaction within an EFIRM plate well. The development of a highly sensitive and specific non-invasive saliva-based neutralizing antibody assay would be of great value for large-scale applications, such as evaluating herd immunity, predicting the efficacy of vaccines, and estimating the requirement for booster doses. EFIRM NAb assay is a versatile platform that can easily be adapted to the detection of NAbs against the very-evolving SARS-CoV-2 virus. By understanding how well the vaccine-

induced antibodies neutralize various strains, health authorities can make informed decisions regarding the potential need for additional doses or the development of variant-specific vaccines.

As COVID-19 rapidly emerged and triggered a global pandemic, the infrastructures of intensive care units and diagnostic laboratories faced an abrupt surge in patients. This influx placed a significant strain on the resources of these facilities, surpassing their previously anticipated capacities. Even with the presence of vaccines, COVID-19 is expected to remain a significant challenge in the foreseeable future. Breakthrough infections and ongoing cases of COVID-19 persist, similar to the experiences with diseases like polio, Hepatitis B, and human papillomavirus, where vaccines have not completely eradicated the infections even after several decades. Moreover, if the data suggesting short-lived SARS-CoV-2 antibodies necessitate frequent revaccination, it is unlikely that the uptake will be sufficient, considering the moderate annual flu vaccination rate of approximately 50% in the United States. With the continuous emergence of variants, it has become evident that existing diagnostic testing infrastructure and population surveillance for vaccine effectiveness are insufficient to manage pandemic-scale events. There is an urgent need to address the unmet requirements for enhancing testing workflows and developing technologies capable of managing large-scale crises.

OBJECTIVES AND SPECIFIC AIMS

The goal of this study was to use the EFIRM technology to develop a saliva-based assay for measuring COVID-19 NAbs and compare the level of NAbs in saliva and plasma. We proposed the following specific aims:

1. Develop EFIRM-based neutralizing antibody technical assay for saliva and plasma

- Establish limit of detection (LOD)

2. Validate neutralizing antibody assays with clinical samples

- Establish cutoff value to determine whether a test result is positive or negative
- Compare the EFIRM plasma assay with PRNT and cPass assays
- Establish sensitivity and specificity

3. Submit the data to the data coordination center (DCC)

4. Submit the invention report to UCLA

MATERIALS AND METHODS

Pre-pandemic SMC saliva samples

Saliva was collected from patients admitted to the Samsung Medical Center in Korea between 2014 and 2018. Informed consent was obtained from all participants. Approximately 1 mL of whole saliva was expectorated into a 50cc conical tube placed on ice. The saliva was processed within 30 minutes of collection. Subsequently, the samples were spun in a refrigerated centrifuge at 2,600 xg for 15 minutes at 4 ℃. The resulting supernatant (cell-free saliva) was carefully transferred into a 2 mL cryotube. To preserve the samples, 1 μL of Superase-In (Ambion) was added to the tube, followed by gentle inversion to ensure thorough mixing. The cryotube was then frozen using dry ice and subsequently stored at -80 ℃.

Pre-pandemic plasma samples

Plasma samples collected from healthy individuals prior to 2019 were purchased from innovative research. Donors provided whole blood samples that were collected in K2EDTA tubes. Based on the instructions provided by the vendor, whole blood was subjected to centrifugation at a speed of 5,000 xg for 15 minutes. The resulting plasma was then separated using a plasma extractor.

Outpatient COVID-19 patient samples

Archived saliva and plasma samples from recovered mild COVID-19 patients who had positive qPCR tests for COVID-19 infection were obtained from an ongoing observational study of outpatient COVID-19. Recovered persons who had mild COVID-19 without need for supportive care were recruited for enrollment. At the study visits participants provided blood (for serum, plasma, and PBMC) and saliva samples for a specimen repository. All participants provided informed consent via a UCLA IRB-approved protocol. The participants were subsequently vaccinated while enrolled in the study and provided post-vaccination samples. All the saliva and plasma samples of outpatient COVID-19 cohort utilized in this study were obtained from individuals that had positive PCR test and received one or two vaccinations. Table 1 includes the COVID status and vaccination data of these patients. The saliva sample of patient number #21 was included in the saliva receptor operating curve (ROC) analysis, while the corresponding paired plasma sample was not available. Consequently, we excluded this particular plasma sample from the plasma ROC analysis and any comparison analyses between the plasma and saliva samples. The saliva samples were subjected to centrifugation at 2,600 xg for 15 minutes at 4 ℃. The resulting supernatant, which contains cell-free saliva, was utilized for further analysis. The whole blood was processed into plasma via centrifugation.

Table 1: COVID status and vaccination data of outpatient COVID-19 patient cohort

Number	Days since onset of symptoms	Type of vaccination	Days since 1st vaccination	Days since 2nd vaccination
1	338	Moderna	11	
2	330	Moderna	54	28
3	345	Pfizer	15	
4	87	Moderna	3	
5	98	Moderna	14	
6	330	Moderna	54	28
7	360	Moderna	33	5
8	341	Moderna	8	

Number	Days since onset of symptoms	Type of vaccination	Days since 1st vaccination	Days since 2nd vaccination
9	316	Pfizer	14	
10	331	Pfizer	29	7
11	344	Pfizer	42	20
12	338	Pfizer	8	
13	359	Pfizer	29	8
14	301	Pfizer	21	0
15	126	Moderna	42	12
16	86	Moderna	17	
17	104	Moderna	35	10
18	53	Moderna	18	
19	52	Moderna	28	
20	327	Moderna	12	
21	146	Moderna	45	17
22	442	Pfizer	43	19
23	465	Moderna	75	47
24	299	Pfizer	54	33

Vaccinated (infection naïve) patient samples

Archived saliva and plasma samples from infection naïve vaccinated individuals were obtained from an ongoing observational study at UCLA. Healthy persons without a history of SARS-CoV-2 infection who were undergoing SARS-CoV-2 vaccination (any vaccine) were recruited for enrollment prior to receiving their first vaccine dose, then followed-up after each vaccination and beyond. At each study visit participants provided blood (for serum, plasma, and PBMC) and saliva for a specimen repository. All participants provided informed consent under a UCLA IRB-approved protocol. The saliva and plasma samples utilized in this study were obtained after a minimum of 13 days following the first vaccination. Table 2 includes the vaccination data of these patients. Saliva samples were spun in a refrigerated centrifuge at 2,600 xg for 15 minutes at 4 ℃. The supernatant was used for the development of the assay. The whole blood was processed into plasma via centrifugation.

Table 2: Vaccination data of the vaccinated (infection naïve) patient cohort

Number	Type of vaccination	Days since 1st vaccination	Days since 2nd vaccination
1	Pfizer	32	11
2	Pfizer	32	11
3	Pfizer	45	24
4	Pfizer	33	12
5	Pfizer	42	21
6	Moderna	13	
7	Pfizer	44	21

Design of the EFIRM SARS-CoV-2 neutralizing antibody assay

The EFIRM neutralizing antibody assay is designed to detect functional immunoglobulins that effectively neutralize the interaction between the RBD of the SARS-CoV-2 spike protein and the hACE2 receptor. This assay successfully replicates the virus-host interaction within an EFIRM plate well, enabling precise detection and characterization of functional neutralizing antibodies.

The schematic of the EFIRM SARS-CoV-2 neutralizing antibody assay is shown in Figure 3. Initially, the diluted saliva or plasma samples and controls are pre-incubated with a horseradish peroxidase conjugated RBD (RBD-HRP) for 30 minutes to allow the interaction and binding of neutralization antibodies to RBD-HRP. The principle of the EFIRM platform is that a biomolecule (in this case hACE2 receptor) is added to a liquid pyrrole solution that is then pipetted into the bottom of microtiter wells containing a gold electrode at the bottom of each well. After the solution is added to each well, the plate is placed into the EFIRM Reader and subjected to an electric voltage leading to polymerization. This procedure results in each well becoming coated with a conducting polymer gel containing the hACE2 protein. Following the polymerization, the mixture of samples and controls with RBD-HRP is added to the EFIRM plate and is incubated at 37°C for 15 minutes. The unbound RBD-HRP as well as any RBD-

HRP bound to non-neutralizing antibodies will be captured on the plate. Neutralizing antibodies complexed with RBD-HRP remain in the supernatant and are eliminated during the subsequent washing steps. After the wash steps, TMB solution is added, and after 5 minutes the plate is placed in the EFIRM reader which measures the electric current in the nanoampere (nA) scale. The current of the sample is inversely dependent on the titer of the anti-SARS-CoV-2 NAbs. The instrument possesses the remarkable capability to precisely measure current in the picoampere (pA) range, ensuring that the measurement falls comfortably within the instrument's capacity (40, 42, 43). The utilization of current measurement instead of optical absorbance, as commonly employed in typical ELISA assays, offers two significant advantages over standard ELISA techniques. Firstly, it enables precise quantitation of the antibodies. Secondly, the measurement of current provides increased sensitivity, which is particularly crucial considering that antibody levels in saliva are typically lower than those in plasma (40, 43). The precise details of the assay are described in the next paragraph.

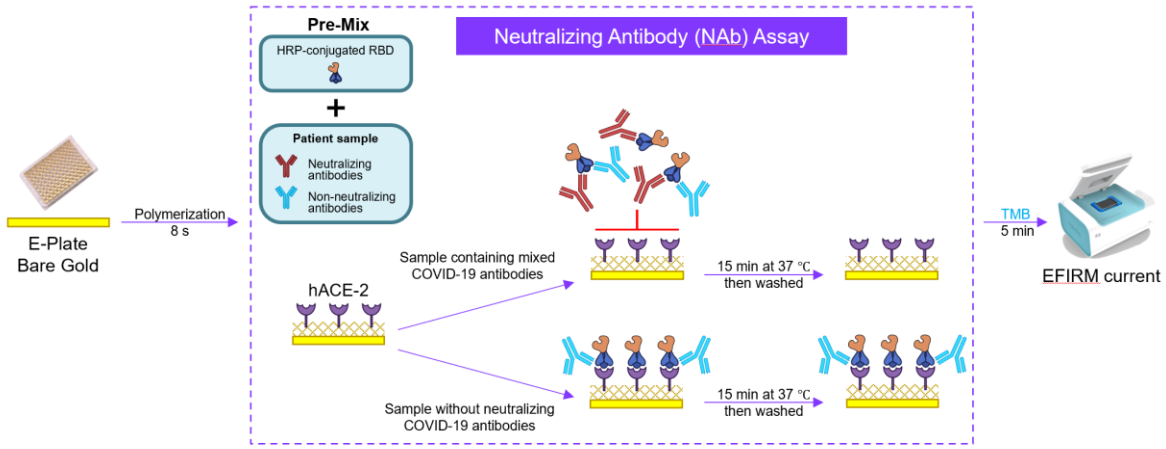

Figure 3. Schema and biorecognition elements of EFIRM SARS-CoV-2 NAb assay

The EFIRM neutralizing antibody assay was developed using hACE2 protein immobilized onto a gold electrode. A mixture of hACE2 protein (GenScript, Piscataway, NJ) was diluted in 1 mL master mix with 5 µL of pyrrole (W338605; Sigma-Aldrich Corp., St.

Louis, MO), 50 µL of 3M potassium chloride, and 945 µL of UltraPure water (Thermo Fisher Scientific, Waltham, MA) and vortexed prior to loading onto each electrode on the 96-well gold electrode plate (EZLife Bio, Woodland Hills, CA). The hACE2 mixture was added to the wells, ensuring that each well contained 500 ng of hACE2. For receptor immobilization, a cyclic square-wave electrode field was applied for 5 cycles of 1 second at 350 mV and 1 second at 950 mV (10 seconds total). After the electrochemical polymerization, each electrode was washed for 6 cycles in PBS-T buffer — 1x phosphate-buffered saline (Affymetrix Inc, Sunnyvale, CA) and 0.05% Tween 20 (Bio-Rad, Hercules, CA) using a 96-channel Biotek 405LS plate washer programmed to aspirate and dispense 400 µL of solution per cycle. Saliva samples were diluted at 1:2, plasma samples at 1:10, and cPass positive and negative controls at 1:10 using a sample dilution buffer (GenScript, Piscataway, NJ). HRP conjugated wild-type RBD was diluted 1:800 with RBD dilution buffer (GenScript, Piscataway, NJ). 60 µL of diluted saliva, plasma, as well as the positive and negative controls, were pre-incubated with 60 µL of diluted RBD-HRP for 30 minutes to allow the interaction and binding of neutralization antibodies to RBD-HRP. 100 µL of the mixture was then added to the EFIRM capture plate pre-coated with the hACE2 protein. All samples and controls were tested in duplicates. If the sample contained SARS-CoV-2 neutralizing antibodies, they would bind to the RBD-HRP during the initial 30 minutes and inhibit the interaction with ACE2. However, if the sample did not contain neutralizing antibodies, the RBD-HRP would bind to the ACE2-coated wells during a 15-minute incubation at 37 °C. Wash step was repeated. Finally, 100 µL of the 3,3,5,5´-tetramethylbenzidine substrate solution (Thermo Fisher Scientific, Waltham, MA) was pipetted to the surface of the electrode, and after 5 minutes incubation at room temperature, the plate was placed into the EFIRM reader where current was measured at -200 mV for 60 seconds (Figure 3). The current in nA was measured 3 times for each well. The process for reading the

entire 96 well plate requires less than 2 minutes. The summary of the assay procedure is shown in Figure 4.

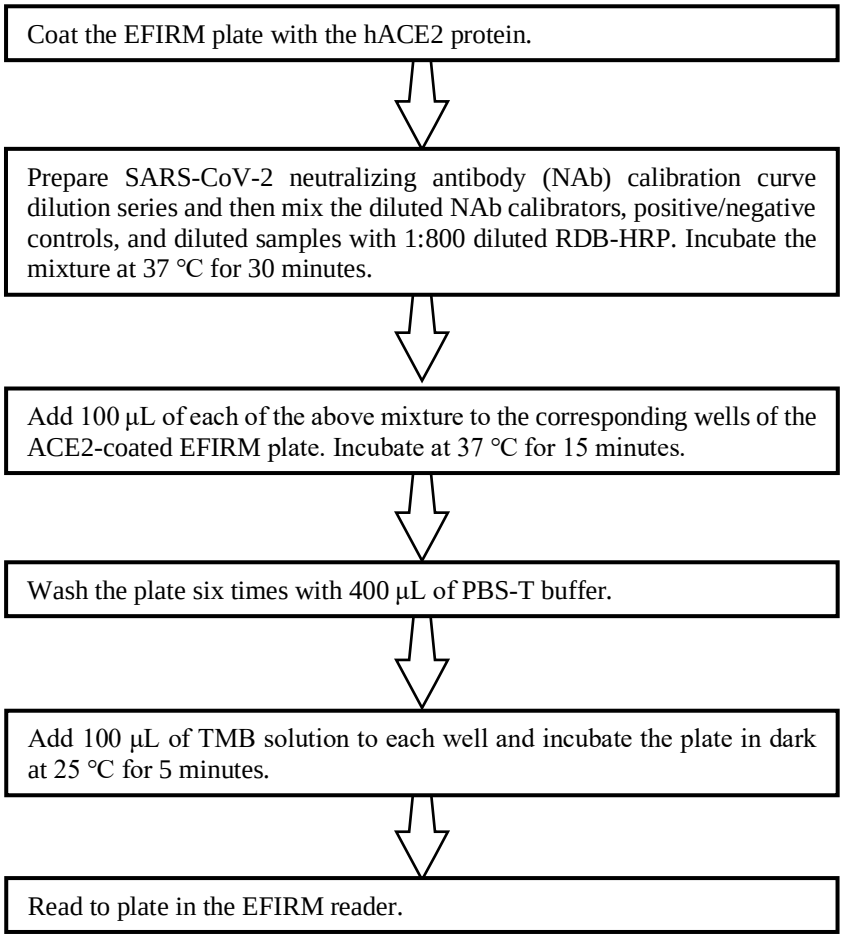

Figure 4: Assay procedure summary

The percent signal inhibition for the detection of neutralizing antibodies was calculated from the formula below.

%Signal Inhibition = (1 – electric current of sample / electric current of negative control) × 100.

The test was calibrated for the quantitative detection of anti-SARS-CoV-2 neutralizing antibodies using the SARS-CoV-2 neutralizing antibody calibrator (GenScript, Piscataway,

NJ). The NAb concentrations were as follows: 300 U/mL, 150 U/mL, 75 U/mL, 37.5 U/mL, 18.75 U/mL, 9.375 U/mL, and 4.688 U/mL. The data generated from the NAb calibration curve was plotted with EFIRM current on the Y-Axis versus concentration on the X-Axis using a 4PL model with GraphPad Prism. The interpolated titer of samples from the standard curve was determined. Quantitative results were expressed in Units/mL. Samples with interpolated values exceeding 200 U/mL were subjected to dilution and reanalyzed using a higher dilution factor. This was done to ensure accurate and within-range measurements for samples with higher concentrations. In order to determine the neutralizing titer per sample, the interpolated titer was multiplied by the corresponding sample dilution factor, taking into account both the initial dilution in sample buffer and the subsequent 1:2 dilution with RBD-HRP.

Optimization of ACE2

To optimize the concentration of ACE2 for each well, we ran the calibration curve dilution series on three different ACE2 concentrations: 300 ng, 400 ng, and 500 ng. Then, we calculated the reduction in the current of each calibrator dilution series from the current of calibrator with zero NAb concentration, as well as the percentage of current reduction. The data generated from the NAb calibration curve was plotted with current reduction and %current reduction on the Y-Axis versus concentration on the X-Axis. Based on the calibration curves, we chose 500 ng ACE2 concentrations to have a better distinction between the different concentrations of the calibrator (Figure 5, Figure 6).

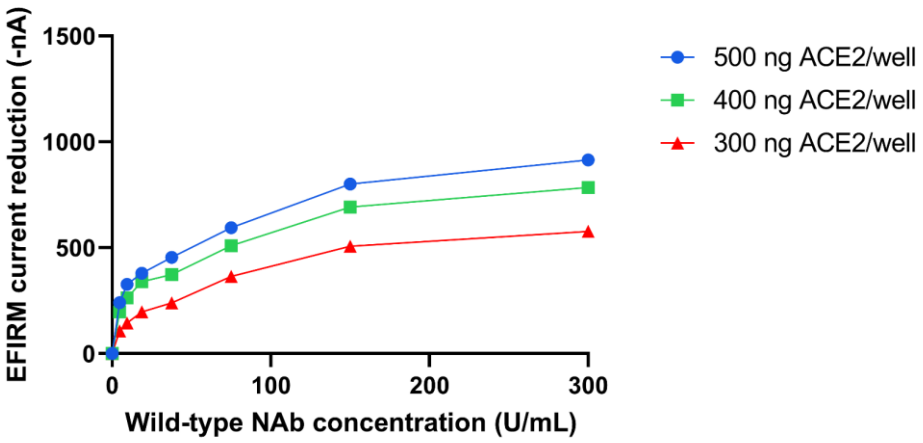

Figure 5: EFIRM current reduction in different ACE2 concentrations

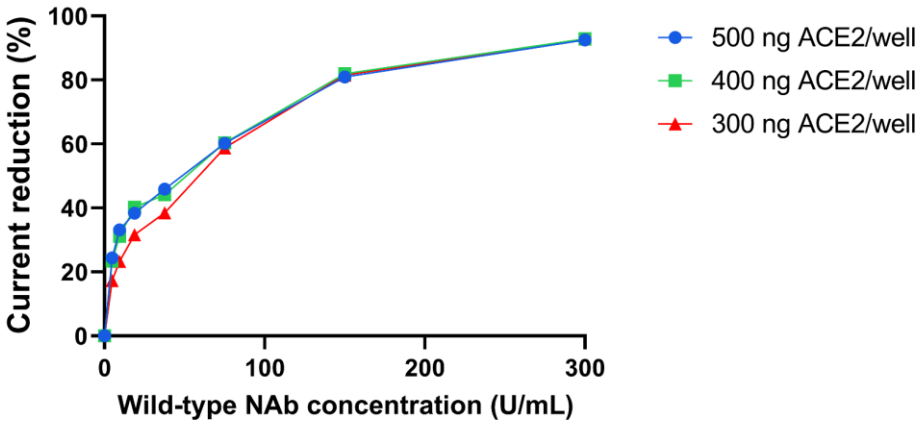

Figure 6: %Current reduction in different ACE2 concentrations

Optimization of RBD-HRP

To optimize the RBD-HRP dilution factor, we mixed the calibration curve dilution series with three different RBD-HRP dilutions: 1:500, 1:800, and 1:1000. The data generated from the NAb calibration curve was plotted with current reduction and %current reduction on the Y-Axis versus concentration on the X-Axis. Based on the calibration curves, we chose 1:800 dilution factor for RBD-HRP (Figure 7, Figure 8).

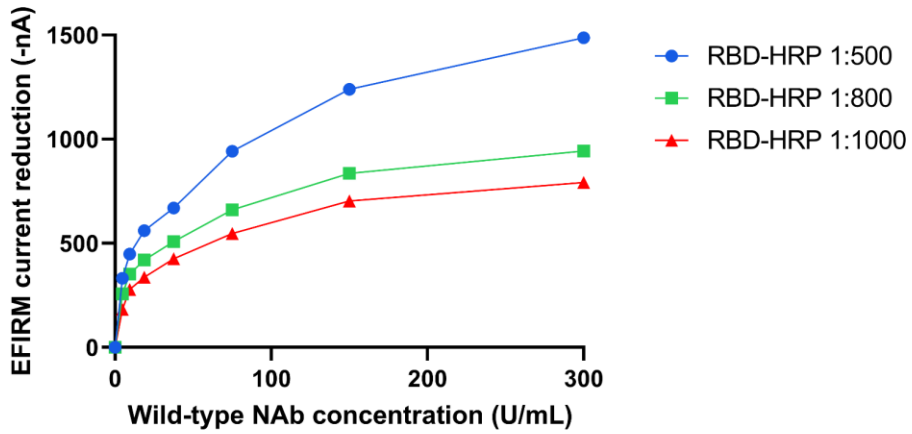

Figure 7: EFIRM current reduction in different dilutions of wild-type RBD-HRP

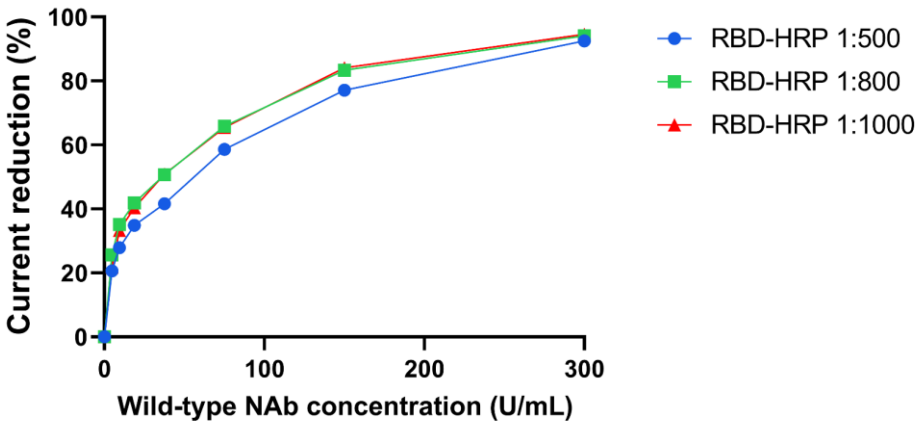

Figure 8: %Current reduction in different dilutions of wild-type RBD-HRP

Plasma cPass SARS-CoV-2 neutralization antibody ELISA test

We purchased an FDA EUA cPass SARS-CoV-2 Neutralization Antibody Detection Kit (GenScript, Piscataway, NJ), which is a blocking ELISA test for detecting functional neutralizing antibodies in plasma. We processed samples exactly as described in the package insert. The cPass assay has a linear quantitative range between 47 U/mL and 185 U/mL. Samples with interpolative titers exceeding 185 U/mL were diluted and ran again in a higher dilution factor.

Analytical performance test

In order to determine the LOD, we designed a comprehensive experiment to assess the repeatability of the assay. We evaluated the effect of operator, components from the kit, EFIRM plate, and days on the baseline EFIRM current. Two different operators independently performed two replicates of negative controls from three different cPass SARS-CoV-2 Neutralization Antibody Detection Kits on three separate EFIRM plates over the course of three days. We conducted calculations on these 108 datasets to determine the mean and standard deviation (STD) of EFIRM current, and subsequently determined the LOD current using the formula: LOD current = mean current – 3 × STD. The data obtained from the NAb calibration curve was plotted using a 4PL model in GraphPad Prism, with EFIRM current on the Y-Axis and concentration on the X-Axis. The LOD U/mL was then calculated using the Prism 4PL model.

Clinical performance test of EFIRM saliva NAb assay

Paired plasma and saliva samples from outpatient COVID-19 and vaccinated (infection naïve) cohorts were assayed for EFIRM NAb. Pre-pandemic SMC saliva and pre-pandemic plasma samples were used to determine the clinical specificity and cutoff.

To validate the clinical performance of the EFIRM saliva NAb assay, we compared 31 saliva samples from outpatient COVID-19 patient cohort and vaccinated (infection naïve) patient cohort (24 outpatient COVID-19 patient samples and 7 vaccinated infection-naïve patient samples) with 60 saliva samples from the pre-pandemic SMC saliva cohort. The calculated inhibition of each sample was used to plot a ROC curve, and the cutoff was studied accordingly. For ROC analysis, saliva samples of outpatient COVID-19 patients and vaccinated (infection naïve) patients were considered true positives, and pre-pandemic saliva samples were labeled as true negatives.

Clinical performance test of EFIRM plasma NAb assay

For clinical validation of the plasma NAb assay, we compared 30 paired plasma samples obtained at the same visit from outpatient COVID-19 patient cohort and vaccinated (infection naïve) patient cohort (23 outpatient COVID-19 patient samples and 7 vaccinated infection-naïve patient samples) with 60 plasma samples from pre-pandemic plasma cohort. The inhibition of each sample was used to plot a ROC curve to determine the cutoff, sensitivity, and specificity. During the ROC analysis of plasma samples, samples obtained before November 2019 (pre-pandemic) were labeled as true negatives. On the other hand, plasma samples from outpatient COVID-19 patient cohort and vaccinated (infection naïve) patient cohort were labeled as true positives.

RESULTS

Specimen characteristics

In the outpatient COVID-19 patient cohort, there was an average of 263 days between the day of symptom onset, 29 days between the first vaccination, and 18 days between the second vaccination and sample collection. In the vaccinated (infection naïve) patient cohort, there was an average of 34 days between first vaccination, and 17 days between the second vaccination and sample collection (Table 1, Table 2).

Development of the SARS-CoV-2 EFIRM NAb test

The EFIRM NAb assay was developed using hACE2 protein immobilized onto a gold electrode. The protein-protein interaction between RBD-HRP and hACE2 is disrupted by NAbs against SARS-CoV-2 RBD, if present in a clinical sample. The current of the sample is inversely dependent on the titer of the anti-SARS-CoV-2 NAbs.

Determination of analytical performance

The effect of different factors including operator, components from the kit, EFIRM plate, and days on the baseline EFIRM current is shown in Figure 9. Our assay demonstrated high repeatability and reproducibility as the results exhibited minimal variation due to different effectors.

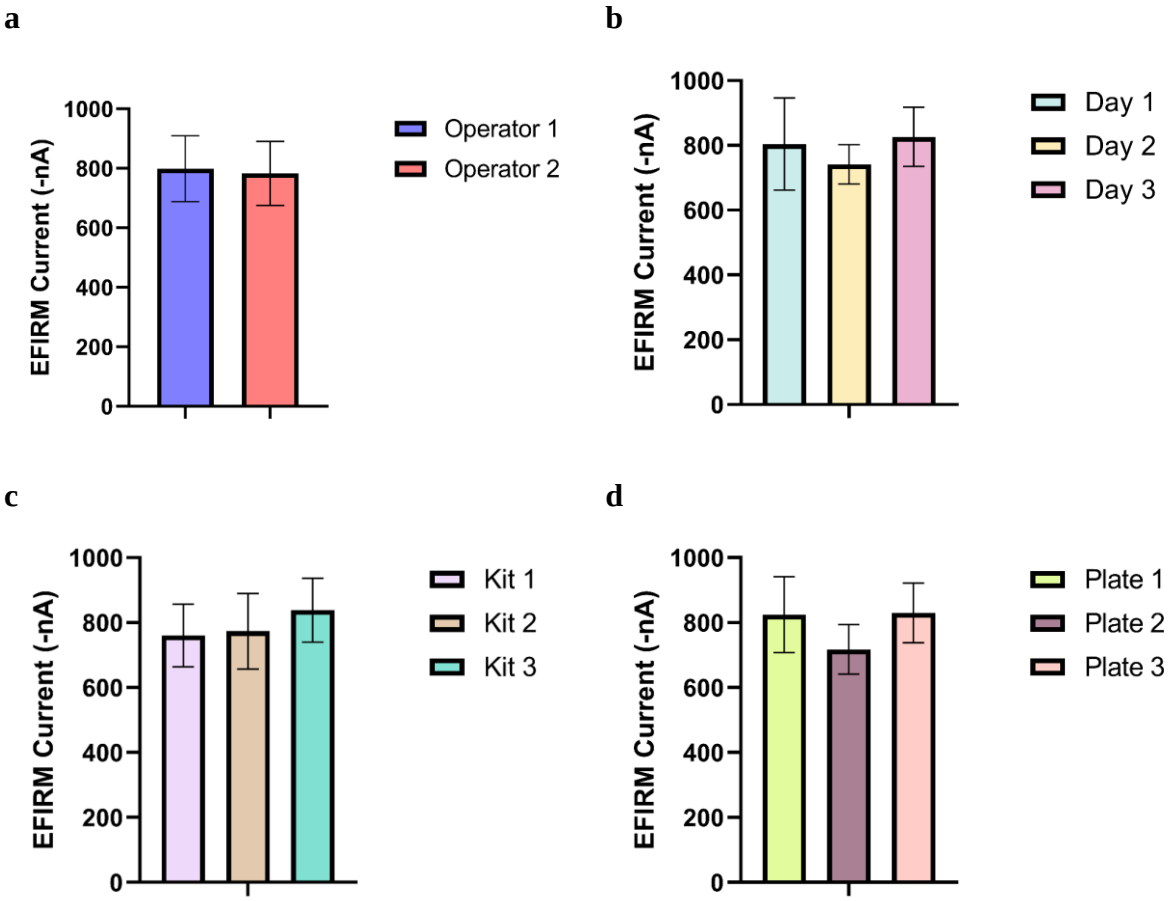

Figure 9: The effect of different factors on the baseline EFIRM current a) effect of operators b) effect of days c) effect of kits d) effect of plates

Based on the LOD current from the repeatability experiment analysis and calibration curve, we calculated LOD as 31.6 U/mL (Figure 10).

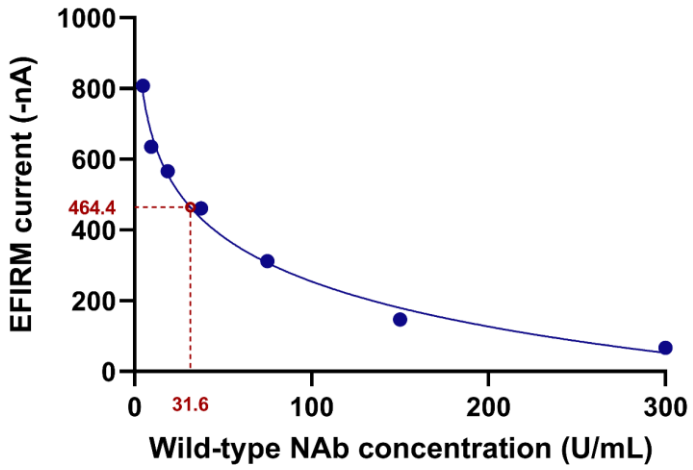

Figure 10: SARS CoV-2 neutralizing antibody calibration curve

Comparison to current EUA test

The cPass SARS-CoV-2 Neutralization Antibody assay has an LOD of 47 U/mL for detecting NAbs (29). In comparison, the EFIRM NAb assay exhibits superior performance with an LOD of 31.6 U/mL that is substantially lower than the cPass assay. Therefore, the EFIRM NAb assay outperforms the cPass Neutralization Antibody Detection assay in terms of its ability to detect NAbs.

Clinical validation of EFIRM NAb test with saliva

The EFIRM saliva NAb assay distinguished outpatient COVID-19 patient samples and vaccinated (infection naïve) patient samples (n = 31) from the healthy group (n = 60) with an AUC of 0.923 (95% CI: 0.869 to 0.976), a sensitivity of 87.10%, and a specificity of 86.67%. The cutoff value for the EFIRM saliva neutralizing antibody assay was determined to be 22% signal inhibition (Figure 11).

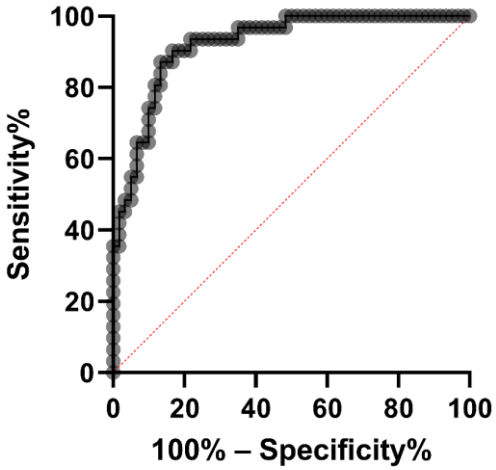

Figure 11: ROC analysis of EFIRM saliva NAb test performance

Clinical validation of EFIRM NAb test with plasma

The EFIRM plasma NAb assay differentiated outpatient COVID-19 patient samples and vaccinated (infection naïve) patient samples (n = 30) from the healthy group with an AUC of 1.000, a sensitivity of 100%, and a specificity of 100%. The determined cutoff value for the EFIRM plasma neutralizing antibody assay was determined as 26.5% signal inhibition (Figure 12)

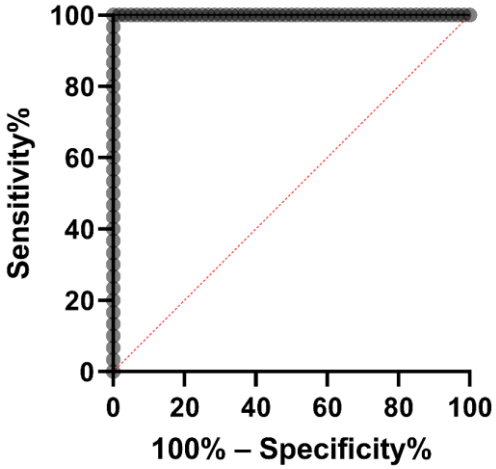

Figure 12: ROC analysis of EFIRM plasma NAb test performance

Clinical agreement between EFIRM plasma NAb assay and PRNT50

To validate the clinical performance of the EFIRM plasma NAb assay a clinical agreement study was conducted using as comparator the Plaque Reduction Neutralization Test (PRNT) utilizing the SARS-CoV-2 virus. The cutoff for the PRNT comparator tests was determined as described in Table 3. The combined cohort comprised samples from normal healthy people (n = 6) and samples from RT-PCR confirmed SARS-CoV-2 positive patients (n = 9). The EFIRM plasma sample results were compared to a Plaque Reduction Neutralization Test performed to WHO guidelines. The EFIRM plasma NAb assay has shown 100% PPA and 100% NPA with the gold standard PRNT.

Table 3: PRNT50 Result Interpretation

Value Result (Dilution Titer)	**Result**	**Test Result Interpretation**
> 1:20	Positive	Neutralizing antibodies for SARS-CoV-2 are detected at 50% viral neutralization.
≤ 1:20	Negative	Neutralizing antibodies for SARS-CoV-2 are not detected at 50% viral neutralization.

Correlation between NAb titers in cPass and EFIRM plasma NAb assays

We assessed the NAb titer in the plasma samples of 30 outpatient COVID-19 patient samples and vaccinated (infection naïve) patient samples. We utilized both the EFIRM plasma NAb assay and the cPass SARS-CoV-2 Neutralization Antibody Detection assay to measure the level of NAbs and compared the results. A strong correlation was found between the NAb titers obtained from the cPass assay and the EFIRM plasma SARS-CoV-2 NAb assay ($r = 0.98$, $p < 0.0001$). Pearson correlation coefficient (r) and p-value are indicated in Figure 13.

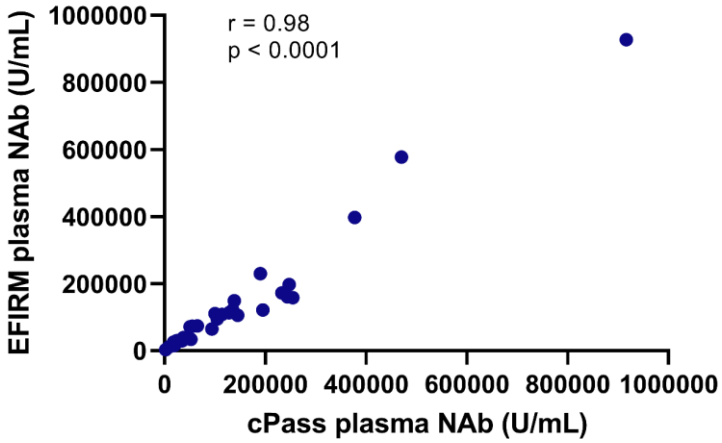

Figure 13: Correlation between SARS-CoV-2 NAb titers in cPass and EFIRM plasma NAb assays

Correlation between NAb titers in plasma and saliva

By utilizing a versatile platform capable of detecting neutralizing antibodies in both saliva and plasma, we were able to evaluate and compare the levels of neutralizing antibodies. A subset of plasma and saliva sample pairs (n = 30) collected from the same patient on the same day from outpatient COVID-19 patient and vaccinated (infection naïve) patient cohorts were used to determine the correlations in levels of neutralizing antibodies. Through the EFRIM saliva and plasma NAb assays, we determined the neutralizing antibody titer in these samples. A significant correlation was observed between the levels of neutralizing antibodies in paired saliva and plasma, emphasizing their interrelationship ($r = 0.75$, $p < 0.0001$) (Figure 14).

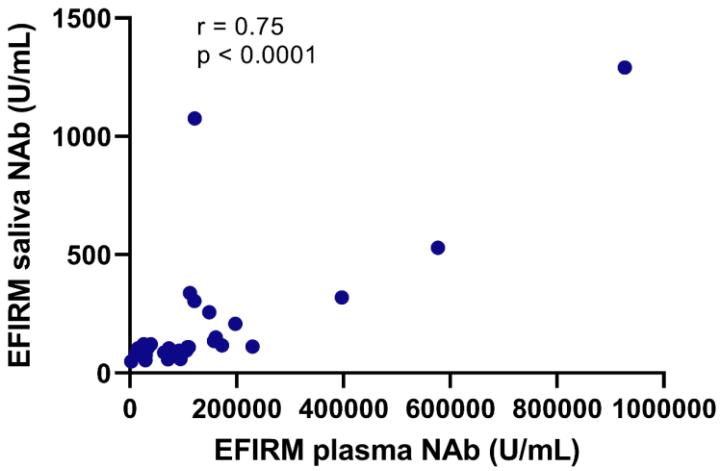

Figure 14: Correlation between neutralizing antibody titers in paired saliva and plasma measured on EFIRM platform

Saliva equivalence of neutralizing activity to SARS-CoV-2 in plasma

We also compared the level of NAbs in the saliva and plasma of the mentioned paired plasma and saliva samples measured on EFIRM and cPass platforms, respectively. The was a significant correlation between the neutralizing antibody titers ($r = 0.77$, $p < 0.0001$) (Figure 15). A recent study estimated that the neutralization level required for 50% protection from SARS-CoV-2 infection equals 54 international units (IU)/mL neutralizing antibodies in plasma (23). GenScript demonstrated that titers interpolated from the cPass calibration curve are converted to WHO IU/mL by multiplying the cPass U/mL titer obtained from the calibration curve generated using the SARS-CoV-2 neutralizing antibody calibrator by a factor of 1.626 (29). Therefore, 54 WHO IU/mL will be equivalent to 33.2 U/mL NAb interpolated from cPass calibration curve which is equal to 664 U/mL total NAbs in the plasma sample considering the sample dilution factor. By employing a second-order local polynomial regression model (in the log scale), we performed interpolation to determine the saliva equivalency of this level of total NAbs in plasma. The interpolated value for this level is expected to be 87 U/mL total NAb in saliva.

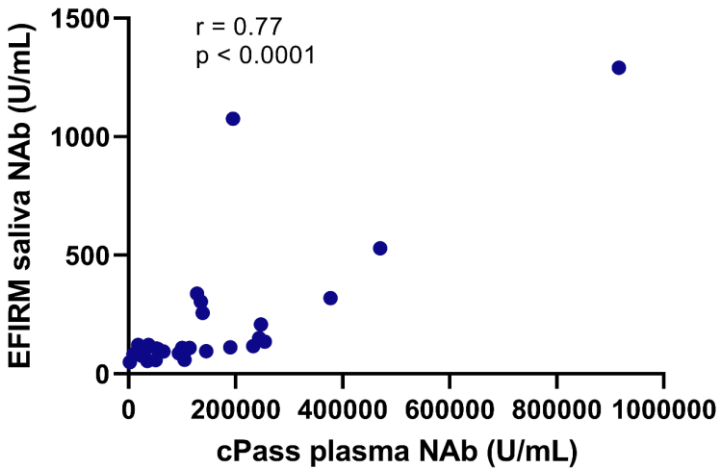

Figure 15: Correlation between neutralizing antibody titers in paired saliva and plasma measured on EFIRM and cPass platforms

DISCUSSION

We have developed a multiplexable, quantitative, non-invasive electrochemical saliva-based assay for SARS-CoV-2 functional NAbs. Our assay stands as the sole testing method capable of accurately measuring neutralizing antibodies in saliva samples. Saliva NAb assay has sufficient sensitivity and specificity to be useful for population-based monitoring and monitoring of individuals following vaccination. In order to enhance specificity and minimize false positives due to technical issues, our non-invasive test provides the option for repeat testing on a second sample. Due to the simplicity of saliva collection, which can be easily performed at home, obtaining a second sample is not a challenging task. However, it is important to note that there remains a possibility of biological false positives, potentially caused by cross-reactivity with other infectious or environmental agents.

To determine whether saliva might be used in a diagnostic test for measuring neutralizing antibodies, we investigated whether the levels of NAbs in saliva correlate with those in plasma. A significant positive correlation was found between neutralizing antibody

titers suggesting that saliva may serve as a surrogate measure of systemic immunity to SARS-CoV-2. NAbs in saliva are most likely to originate from plasma through a filtration process. Therefore, the level of post-immunization neutralizing antibody titers in saliva can be used as an immune correlate of protection for COVID-19 vaccines. It was the first study comparing the level of neutralizing antibodies in saliva and plasma. Comparing the level of binding antibodies in saliva and plasma, previous studies found a positive correlation between the levels of anti-spike IgG and IgM antibodies in serum and saliva. In contrast, the levels of saliva anti-spike secretory IgA antibodies, which are not derived from the circulation but were produced at the mucosa, did not show a strong correlation with serum IgG levels. Therefore, it was suggested that saliva can be a reliable alternative for antibody testing, especially for the measurement of anti-spike IgM and anti-RBD IgG (40, 41, 44).

Numerous studies have consistently shown a strong correlation between the in vitro neutralization titer and both vaccine efficacy and an individual's protection against symptomatic SARS-CoV-2 infection. While neutralizing antibody levels are a reliable indicator of protection, determining a specific threshold for protection using serologic tests is challenging. This is primarily due to the absence of a defined threshold; instead, there exists a gradient of vaccine efficacy that increases with neutralization. Additionally, several significant challenges hinder the establishment of a specific threshold for high protection based on an individual's neutralization titer. These challenges include the diversity of assays used to measure neutralization, the complexity of translating neutralization levels across different assays, the continuous emergence of new and more resistant variants, and the uncertainties associated with estimating individual neutralization titers. These factors collectively contribute to the difficulty in identifying a precise neutralization threshold for assessing high protection against COVID-19 (23, 26-28).

This assay presents several advantages compared to other saliva antibody tests. It assesses the interaction between the hACE2 receptor and the RBD, providing insights into the neutralizing function of antibodies. The test can indirectly detect immunoglobulins that disrupt the RBD-hACE2 interaction, without being limited to a specific antibody isotype (e.g., IgG, IgM, or IgA).

Moreover, this assay can detect neutralizing antibodies specific for blocking different variants of SARS-CoV-2 (such as wild-type, Delta, etc.) on the same plate only by mixing the samples with purified RBDs of different variants. By detecting neutralizing antibodies specific to each variant, our assay can provide valuable information about the immune response generated by vaccination. This data can help evaluate the vaccine's ability to elicit neutralizing antibodies against different variants and inform decisions regarding booster shots or the need for variant-specific vaccines.

The EFIRM NAb test is designed for high-throughput analysis and utilizes a plate-based format. The entire plate can be processed within 1.5 hours, resulting in a quick turnaround time once the sample reaches the laboratory. Automation of the assay is easily achievable with standard liquid handlers, allowing for extremely high throughput since the EFIRM reader is only necessary for a brief polymerization step at the beginning of the assay, which takes less than a minute, and a 3-minute measurement phase at the end of the assay.

Most importantly, the NAb assay can be integrated with EFRIM RNA, antigen, and binding antibody assays that we previously developed. The clinical performance of EFIRM’s detection of SARS-CoV-2 compared to approved EUA assays for gRNA, antigen, antibodies and neutralizing immunity is shown in Table 4. 40 μL of saliva is sufficient for EFIRM to concurrently detect all 4 dimensions of SARS-CoV-2 information, directly, non-invasively with a performance that surpasses current EUA approved assays. This integrated approach

would enable a comprehensive understanding of the infection, infectivity stages and host immune response, facilitating more accurate diagnostic and therapeutic decision-making.

Table 4. Performance of EFIRM saliva RNA, antigen, binding antibody, and functional neutralizing antibody assays compared to EUA authorized tests

Assay	**LOD**	**Sensitivity**	**Specificity**	**Singular EUA Test (LOD or sensitivity)**	**Comparison to EUA Tests**
vRNA	100 copies/reaction	90% (9/10) (≤15 days post sx)	100% (33/33)	100 copies/reaction (SalivaDirect)	1X
Antigen	3.5 $TCID_{50}$/mL	100% (10/10) (≤15 days post sx)	100% (33/33)	22.5 $TCID_{50}$/mL (Nasal swab)	7X
Combined IgG/M/A Antibody	39 pg/mL	95% (33/35)	100% (81/81)	86-100% IgM serology; 90-100% IgG serology; No EUA IgA serology available	1X to serology assays. No saliva EUA tests available
Neutralizing antibody	31.6 U/mL	87.10% (27/31)	86.67% (52/60)	no EUA saliva neutralizing antibody available	no EUA saliva neutralizing antibody available

The multidimensional EFIRM saliva test not only enables rapid and accurate detection of the current pandemic-causing virus but also lays the foundation for tackling potential future pandemics since it is easy to rapidly develop EFIRM tests to any antigen. Its versatility in detecting multiple biomarkers in a small amount of saliva positions it as a valuable tool for the early identification and monitoring of emerging infectious diseases, allowing for timely containment measures and targeted interventions. This diagnostic platform has the potential to revolutionize future pandemic preparedness and response strategies, enabling swift and effective containment of novel pathogens.

Limitations of the study

This study has a few limitations that should be considered. Firstly, the sample size was relatively small, indicating the need for larger studies to confirm the reproducibility of the

findings. Secondly, the cohorts used in the analysis were from two different countries, serving as the pre-pandemic and outpatient COVID-19 patient and vaccinated (infection naïve) patient cohorts. Ideally, it would have been preferable for the cohorts to come from the same country to minimize potential confounding factors. Furthermore, it was necessary to establish our assay using standard operating procedure (SOP) samples as our pre-pandemic samples were SOP processed. Nevertheless, our assay shows promising prospects in utilizing whole saliva samples, which could enhance both sensitivity and specificity. Lastly, the saliva equivalency data of plasma protection level should be applied with caution since we extrapolated the neutralizing antibody titers.

CONCLUSION

In this study, a multiplexable, quantitative, non-invasive electrochemical saliva-based assay was developed to measure SARS-CoV-2 NAbs. We found that saliva is a good matrix for detection of neutralizing antibodies and NAb responses in saliva may serve as a surrogate measure of systemic immunity to SARS-CoV-2. EFIRM saliva NAb assay has sufficient sensitivity and specificity to be useful for population-based monitoring and monitoring of individuals following vaccination.

DISCLOSURES

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