In light of the swift outspread and considerable mortality, coronavirus disease 2019 (COVID-19) necessitates a rapid screening tool and a precise diagnosis. Saliva is considered as an alternative specimen to detect the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) since the viral load is comparable to what are found in a throat and a nasal cavity. The electrical double layer (EDL)-gated field-effect transistor-based biosensor (BioFET) emerges as a promising candidate for salivary COVID-19 tests due to a high sensitivity, a portable configuration, a label-free operation, and a matrix insensitivity. In this work, the authors utilize EDL-gated BioFETs to detect complementary DNAs (cDNAs) and viral RNAs with various testing conditions such as switches of probes, temperature treatments, and matrices. The selectivity is confirmed with cDNA and noncomplementary DNA (ncDNA), exhibiting an eightfold difference in electrical signals. The matrix insensitivity is evaluated, and BioFETs successfully validate the detection of SARS-CoV-2 N-gene RNA down to 1 fm in diluted human saliva with a 95°C- and a 25°C-treatment, respectively. This proposed system has a high potential to be deployed for an on-site COVID-19 screening, improving the disease control and benefitting frontline healthcare system.

1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has rooted in the globe since the first quarter of 2020.[1–3] The epidemiology reports, released by the World Health Organization,[4] confirmed that the spread of the coronavirus disease 2019 (COVID-19) has reached over 177 million people as of late June, 2021. Excluding the unaccounted infections of suspected individuals across 220 countries and territories, the death toll has surpassed 3.85 million. With such a swift outspread and an escalating mortality, an effective tool should be implemented for accurate diagnoses, early detection, and disease control.[5] Since most of the symptoms expressed by the carriers are nonspecific (while some are entirely asymptomatic), symptom-based diagnoses have faced practical challenges.[6,7] Several techniques, which were approved by the Emergency Use Authorizations (EUAs), are currently being deployed to address an increased demand for rapid screenings:[8] for example, i) real-time reverse-transcription polymerase chain reaction (real-time RT-PCR);[9] ii) reverse transcription loop-mediated isothermal amplification (RT-LAMP);[10,11] iii) lateral flow assays;[12,13] and iv) enzyme-linked immunosorbent assay (ELISA).[14]

Real-time RT-PCR is the primary method used for nucleic acid-based detection,[15,16] while COVID-19 sample collection majorly relies on nasal swabs that is particularly uncomfortable for examinees.[17–19] The process is time-consuming (turnaround time: =24 h),[20] and the test requires highly-trained professionals to operate in centralized laboratories, limiting the number of tests measured in a single run.[21,22] Reverse transcription loop-mediated isothermal amplification (RT-LAMP) exhibits a sensitivity of 97.5% and a specificity of 99.7%,[23,24] yet complex protocols and primer design fall short in its commercial applications.[25,26] As an alternative, several antigen tests are in development, such as laboratory-based ELISA,[27] chemiluminescent immunoassay (CIA),[28] and point-of-care (PoC)-based lateral flow technology.[29] However, the accuracy of antigen tests could be compromised due to nonspecific-binding that increases cross-reactivity and leads to false results.[30,31]

To reduce the risk of exposure to COVID-19 among frontline medical workers, the passive drool method can minimize a necessary contact when collecting a sample.[32] Reportedly, the true positive rate (TPR) of COVID-19 samples collected
from saliva was 88.09%; while the TPRs obtained from the specimens of throat swabs and nasal swabs were 34.78% and 65.22%, respectively.\cite{33,34} The viral load found in COVID-positive saliva samples varies from $10^4$ to $10^8$ copies/mL at which real-time RT-PCR needs multiple rounds of amplification to surpass the cycle threshold (Ct).\cite{35,36} To necessitate rapid and precise screenings for COVID-19, field-effect transistor-based biosensors (BioFETs) have emerged as a potential tool amongst novel biosensing techniques.\cite{37–49} The electrical double layer (EDL)-gated BioFET was developed to avoid the Debye screening effect and to enable a detection in a physiological condition.\cite{39–41} The sensing modality was predicated on EDL capacitance and non-faradic effect, resulting in an ease of sample pretreatment. Considering the above advantages, an EDL-gated BioFET has successfully demonstrated molecular assays and cellular monitoring in various matrices such as PBS, serum, whole blood, etc.

In this work, we utilized EDL-gated BioFETs to rapidly detect SARS-CoV-2 viral RNA in saliva without any complex pretreatment (Figure 1). The ssDNA probes were immobilized on the sensor surface, following which the complementary DNA (cDNA) and viral RNA were investigated. A binding of probe/target caused an electrical redistribution in EDL capacitance, leading to the change of FET drain current ($I_d$). The theoretical model demonstrated for nucleic acid hybridization on an EDL-gated BioFET was discussed. The effectiveness of surface functionalization was examined and compared, and 10 µm of probe concentration was selected for the standard condition in this work. The selectivity between cDNA and noncomplementary DNA (ncDNA) was confirmed, and viral RNA detection was scrutinized with varying probes using a 95°C-treatment. Lastly, the detection of SARS-CoV-2 N-gene RNA was successfully validated in diluted human saliva at concentrations ranging from 1 fm to 1 pm, and matrix insensitivity was evaluated. This study particularly aimed to develop a saliva-based viral RNA detection tool using BioFETs where COVID-19 screening could be performed with an uncompromised sensitivity, a rapid readout, and a high accuracy.

2. Experimental Section

2.1. EDL-Gated BioFET Platform

The BioFET platform (Figures 1 and 2) had the extended-gate configuration where the gate terminal of an FET was wire-connected to a detachable sensor stick via gold fingers. A sensor stick (Figure 2b) consisted of 8 individually addressable sensors arranged in a 1 × 8 array. Each sensor had a pair of gold electrodes where the input electrode was supplied with the recurring gate voltages ($V_g$) and the extended-gate electrode was connected to the gate terminal of an FET. A sensing area (500 x 500 µm²) on each electrode was defined by photolithography. A testing solution was drop-casted on a sensor stick, covering all the sensing areas. A signal was read out every 2 min, and 11 measurements were taken for each concentration of the analyte.

The portable reader, implemented with 8 N-channel depletion-mode MOSFETs (Microchip Technology, #LND 150), was used to measure sensor signals as shown in Figure 2c. A pulse of a 4-ms duration was applied at $V_g = 0$ V and $V_g = 1$ V (Figure S1, Supporting Information) with a constant drain-source voltage ($V_d = 2$ V). The characteristic current ($I_d$), calculated by the $I_d$ difference between $V_g = 1$ V and $V_g = 0$ V, was taken as an output. The $I_d - V_d$ characteristics (Figure 3a) of the MOSFET at different $V_d$ were measured by a semiconductor analyzer (Agilent, #B1500A). The transfer characteristics (at $V_d = 2$ V) are shown in Figure 3b, and the maximum transconductance is achieved near $V_g = 0$ V.

![Figure 1. Schematic illustration of the saliva-based COVID-19 viral RNA tests using an EDL-gated BioFET. A saliva sample is collected by the passive drool method and is diluted with a buffer solution. This testing sample is then drop-casted on a sensor stick where the probes are immobilized.](image-url)
2.2. Surface Functionalization

An SU8-passivated sensor stick was exposed to O₂ plasma, and optical images were taken to ensure a proper cleaning. Three primer sequences (Genomics, Taiwan, #N_Sarbeco_CP1, #N_Sarbeco_CF1, and #N_Sarbeco_R1), used in RT-PCR by Taiwan Centers for Disease Control (TCDC), were immobilized on sensor surfaces serving as probes. A fluorescent dye, 6-carboxyfluorescein (6-FAM), was attached at the 3’ end of these sequences (3’FAM) where the dye-tagged complex was excited by a fluorescence microscope and emitted a characteristic color in green. The desired concentrations of each single-stranded DNA (ssDNA) probe were prepared in Tris-EDTA (TE) buffer solution which comprised tromethamine (Tris) (AMRESCO, #0826) and ethylenediaminetetraacetic (EDTA) (Sigma-Aldrich, #ED). 1 mm of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) solution (Sigma-Aldrich, #C4706) was added to each solution, allowing a reaction for 30 min at room temperature. Thiol groups were formed on ssDNA by the reduction of disulfide linkage. A testing solution, in the presence of the ssDNA probes, was drop-casted on an O₂ plasma-cleaned chip and was incubated for 24 h at 4 °C. Gold-thiol bonds were formed, and the ssDNA probes were immobilized on a sensor surface. Later, the unbound probes were removed by a gentle wash with TE buffer solution. The details of the DNA probes are listed below:

- Probe-1: 5’-TTTTTTTGGCAATGTTGTTCCTTGAGGAAGT-3’FAM
- Probe-2: 5’-TTTTTTGTGTAACCGTGGGCGTTAG-3’FAM
- Probe-3: 5’-TTTTTTGAGGAACGAGAAGAGGCTTG-3’FAM

2.3. COVID-19 Viral RNA and DNA

The desired concentrations of cDNA strands (DNA-1, DNA-2, and DNA-3) (Tri-I Biotech, Taiwan, #C-19 N-2, #C-19 N-1, and #C-19 N-3), COVID-19 N-gene viral RNA (1260 bases) (ANTEC Bioscience, Taiwan, #2019-nCoV-N), and COVID-19 S-gene viral RNA (1108 bases) (ANTEC Bioscience, Taiwan, #2019-nCoV-S) were prepared with the testing media which were used for subsequent assays. The media varied from TE buffer solution, diethyl pyrocarbonate (DEPC)-treated water (Sigma-Aldrich, #d5758), human saliva, to sodium dodecyl sulfate (SDS)-treated human saliva. The sequences of the viral RNA are provided in Figure S2 and S3, Supporting Information, and the sequences of the cDNAs are listed below:

- DNA-1 (complementary to Probe-1): 5’-GCTACAACTTCCTCAAGGAACAACATTGCCAAAA- GGCTTCTACGCAGAAG-3’
- DNA-2 (complementary to Probe-2): 5’-GCTACAACTTCCTCAAGGAACAACATTGCCAAAA- GGCTTCTACGCAGAAG-3’
- DNA-3 (complementary to Probe-3)
3. Results and Discussion

The details of the equations used to elucidate the sensing modality of an EDL-gated BioFET can be found in Supporting Information. When a sensing electrode is functionalized with DNA probes (Figure 4a, Supporting Information), the surface charge gets redistributed, \[ \Delta V_{g,(1)} \] and the potential change across a testing solution \( \Delta V_{g,(2)} \), due to the probe functionalization, are formed; and the output of \( I_d \) is measured accordingly by a potential change at the extended electrode \( V_{g,(2)} \). Likewise, the surface charge is redistributed again after the probe-target hybridization, and the corresponding voltage drop/increase \( V_{g,(2)} \) is reflected on a BioFET signal.

3.1. Surface Functionalization and cDNA Detection in TE Buffer Solution

To maximize the efficiency of target captures, an optimal coverage of the immobilized probes should be achieved. The sensor surfaces were functionalized with various concentrations of the ssDNA (Probe-1), ranging from 5 to 20 \( \mu \)m, that were prepared in TE buffer solution. Prior to running an assay, the immobilization of Probe-1 was verified with fluorescent imaging as shown in Figure 4a. A brightness indicated the relative amount of the immobilized probes where the fluorescent dyes emitted green light (\( \lambda = 519 \) nm). The mean fluorescence intensities (MFIs) were analyzed and calculated by using ImageJ (Figure 4b). The error bars represented one standard deviation (\( \sigma \)) of MFI uncertainty measured from 8 subregions of two electrodes of a sensor surface. As Probe-1 was drop-casted with the increasing concentrations, the MFIs increased proportionally. The MFIs were 4.57 RFU, 18.13 RFU, 43.03 RFU, and 86.34 RFU at [Probe-1] = 0, 5, 10, and 20 \( \mu \)m, respectively.

To investigate the BioFET responses with various surface conditions in TE buffer solution at 25 °C, the functionalized sensors detected the target sequence of the cDNA strands. While a non-functionalized sensor, where 0 \( \mu \)m of [Probe-1] was presented, served as a negative control to ensure that there was no significant nonspecific-binding on a sensor surface. 70 \( \mu \)L of different cDNA samples ([DNA-1] = 1 fm, 10 fm, 100 fm, and 1 pm), the substitutes for the viral RNA, were drop-casted and tested. The baseline of each probe concentration was defined as an average of \( I_c \) measured in the absence of target ([DNA-1] = 0 fm). The change of \( I_c \), compared to a baseline, was taken as a sensor signal. Throughout the article and figures, all the error bars of BioFET detections represent \( \sigma \) of uncertainty (n = 7) measured by a sensor. The sensor was washed with TE buffer solution before adding the next cDNA sample at an increasing concentration. As shown in Figure 5a, the control ([Probe-1] = 0 \( \mu \)m) owned variations within \( \pm 5 \) \( \mu A \) across all the cDNA concentrations; while signals of the experiment group, ranging from 5 to 20 \( \mu \)m of [Probe-1], exhibited a positive dependence on the added concentrations of DNA-1. The signal had the most linear response to \( \log([DNA-1]) \) at [Probe-1] = 5 \( \mu \)m, and a higher probe concentration led to a stronger signal where 10-\( \mu \)m probe had 1.5 \times the amplitude of 5-\( \mu \)m probe in the presence of 1-pm DNA-1. However, the high probe concentration did not significantly benefit the signal at [DNA-1] of 1 pm (Figure 5b) where 10-\( \mu \)m probe and 20-\( \mu \)m probe had signals of 45.29 and 47.43 RFU, respectively. Compared to Figure 4a, 20-\( \mu \)m probe might form multiple layers rather than a uniform coverage at the sensor surface. Considering the signal amplitude and linearity, a 10-\( \mu \)m probe was chosen as the optimal condition for the surface functionalization throughout this work.

3.2. cDNA Detection in Diluted Saliva

After the cDNA tests were examined in TE buffer solution, the sensing effectiveness in a biological medium was investigated.

![Figure 4](https://www.advancedsciencenews.com)  a) Fluorescent images of surface functionalization. The ssDNA probes, which were labelled with fluorescent dyes, were immobilized on the sensor surface. b) Measured data of mean fluorescence intensities (MFIs). MFIs were calculated by ImageJ, and error bars represent \( \sigma \) of uncertainty measured from 8 subregions of a sensor surface.
Human saliva was collected from COVID-negative individuals by the passive drool method and was stored in $-20^\circ$C for the subsequent experiments. To reduce the salivary viscosity, the collected saliva was diluted (at a volume ratio of 1:1) with the TE buffer solution prepared for DNA measurements and with the DEPC-treated water prepared for RNA measurements, respectively. An addition of DEPC-treated water reduced the risk of RNA degradation. Following the same procedure as described in the previous section, only the medium was replaced with the diluted saliva. The BioFET signals using different DNA targets were investigated. In the controlled experiment, a non-functionalized sensor was tested and the real-time data can be found in Figure 6a. The uncertainties (1σ) measured in saliva were in the range of 1–1.7 µA, similar to those measured in TE buffer solution. As shown in Figure 6b, an EDL-gated BioFET validated cDNA detection in diluted saliva. The signal amplitude (at [DNA-1] = 1 pm) was 44.28 µA, that is, 0.98x of the signal which was measured in TE buffer solution. To verify the

![Figure 5](image)

Figure 5. a) Measured BioFET signal. The measurements were taken in TE buffer solution at 25 °C. To illustrate the baselines defined in each measurement, the datapoints of 0 fm are presented in the figure. b) Signals at [DNA-1] = 1 pm. 10-µm Probe-1 and 20-µm Probe-1 have a similar outcome.

![Figure 6](image)

Figure 6. cDNA detection measured in diluted saliva at 25 °C. a) Measured real-time data of the controlled experiment. b) Measured BioFET signal of cDNA detection. c) Measured data showing a hybridization selectivity at 1 pm of the tested DNAs.
hybridization selectivity, DNA-2 and DNA-3 (noncomplementary to Probe-1) were tested and the sensor responses were compared at 1 pm of the DNAs (Figure 6c). The group of ncDNAs, DNA-2 and DNA-3, showed responses between 2.43 and 4.86 µA; while the signal of the cDNA (DNA-1) exhibited at least an eightfold increase. The results indicated that 10-µm Probe-1 enabled highly selective probe-target hybridization in human saliva at room temperature.

3.3. COVID-19 Viral RNA Detection with a 95°C-Treatment and Comparison of Various Probes

Following the same procedures (as described in Section 3.1 and 3.2), Probe-2 and Probe-3 were examined at the probe concentrations of 10 µm. The testing conditions were expanded. In the 25°C-treatment group, each kind of probe was immobilized to detect its cDNA (e.g., Probe-1/DNA-1, Probe-2/DNA-2, and Probe-3/DNA-3) in TE buffer solution. In the 95°C-treatment group, each kind of probe was immobilized to detect COVID-19 viral RNA (N-gene) in diluted saliva. The cDNA dataset of Probe-1 was obtained from the measurement of 10-µm probe that has been shown in Figure 5a. To avoid the formation of secondary structure at room temperature,[52] RNA samples were heated for 5 min at 95 °C prior to being drop-casted onto the sensors which were respectively functionalized with Probe-1, Probe-2, and Probe-3. The sensors, in the presence of the heated samples, gradually cooled down in 20 min while signals were recorded simultaneously.

In the group of Probe-1 (Figure 7), both RNA and cDNA can be detected with a similar signal response. The ratio of RNA signal to cDNA signal \( \frac{\text{Amplitude}_{\text{RNA/saliva/95°C}}}{\text{Amplitude}_{\text{cDNA/TE/25°C}}} \) at 1 fm of the nucleic-acid targets was 1.62, while the ratios varied from 0.93 to 1.07 at target concentrations ranging from 10 fm to 1 pm. The trends were significantly different among other probes. The signal ratios of Probe-2 varied from –4.43 to –2.03, while Probe-3 was unable to capture and detect the cDNA (DNA-3) with a 25°C-treatment due to the negligible responses dwelling in the variation of the controlled experiment (±5 µA). Compared to RNA detection using Probe-1, the group of Probe-3 gave rise to the smallest signals (<30 µA) at each concentration while the group of Probe-2 exhibited intermediate signals (<38 µA) with the largest uncertainties (1σ). The pairs of Probe-2/cDNA-2 and Probe-3/cDNA-3 were not capable of proof-of-concept experiments with a 25°C-treatment due to, supposedly, the formation of secondary structure by which the hybridizations were hindered. Secondary structures would be found in ssDNA/RNA molecules due to a self-complementarity, resulting in the formation of hairpin loops.[53,54] These loops act as partial dsDNA and do not actively bind to the complementary strands under normal physiological conditions; therefore, a probe-target binding is hampered. Using an online oligonucleotide properties calculator, we found that DNA-2 had the highest probability (out of the three probes) to form a secondary structure, and DNA-3 had a higher probability than DNA-1 did. As a result, Probe-1 consistently demonstrated the best stability and performance in both cDNA detections with a 25°C-treatment and COVID-19 viral RNA detections with a 95°C-treatment.

3.4. COVID-19 Viral RNA Detection at Room Temperature and Matrix Insensitivity

To validate rapid COVID-19 screenings for on-site applications, BioFETs examined RNA samples in various matrices at room temperature. Following the same procedures as described in the previous sections, Probe-1 (which had a better signal outcome compared to the other two probes) was immobilized to test COVID-19 viral RNAs. SARS-CoV-2 S-gene RNA served as a negative control and was tested in diluted human saliva, while SARS-CoV-2 N-gene RNA was tested with varying media such as diluted human saliva, SDS-treated saliva, and DEPC-treated water. SDS is an ionic detergent commonly used for lysing a viral envelope and intervening in a nucleic acid-protein interaction by denaturing the protein.

Compared to the ncDNA groups shown in Figure 6, BioFETs exhibited larger uncertainties (1σ) (3.56–5.87 µA) with S-gene RNA in the controlled experiment (Figure 8). The phenomenon
can be attributed to the increasing cross-reactivity between Probe-1 and S-gene RNA. Due to a partial complementarity ($\approx 4–8$ nt) between Probe-1 and S-gene RNA sequence, this cross-reactivity could possibly increase false-positive results. As such, an improved probe design (which is more specific to the target) is expected to alleviate cross-reactivity before moving toward clinical trials.

In the positive group, N-gene RNA demonstrated the highest sensitivity in diluted human saliva, and the signal reached 42.14 $\mu$A at a concentration of 1 pm. As such, the signal obtained from diluted human saliva was taken as the benchmark. Smaller signals were found in DEPC-treated water and SDS-treated saliva, and this trend was more obvious at concentrations of 1 fm making them indistinguishable from the negative group (S-gene RNA). While, at RNA concentrations between 100 fm and 1 pm, the samples of DEPC-treated water exhibited $\approx 0.9$ times the signal amplitude of the diluted human saliva group, and the samples of SDS-treated saliva exhibited 0.8 to 0.9 times the signal amplitude (of the diluted human saliva group). The result indicated the matrix-insensitivity prevailed at the higher concentrations (100 fm–1 pm) when detecting SARS-CoV-2 N-gene RNA at room temperature; furthermore, the addition of SDS did not affect the signal significantly. Taken together, EDL-gated BioFETs demonstrated the ability for rapid COVID-19 viral RNA screening using salivary samples with ease of pretreatment, and the portable platform can make the turnaround time less than 1 h.

4. Conclusion

We successfully developed a saliva-based screening tool for COVID-19 viral RNA tests using an EDL-gated BioFET. The assay was conducted on a portable platform where the extended-gate configuration prevented corrosion and prolonged a lifetime. An EDL-gated BioFET was functionalized with ssDNA probes, and a signal was retrieved by an EDL capacitance. The cDNAs and viral RNAs were tested with various testing conditions such as switches of probes, temperature treatments, and matrices. The COVID-19 tests using BioFETs were successfully validated for SARS-CoV-2 N-gene RNA with a 95°C-treatment and a 25°C-treatment, respectively. The results indicate that an EDL-gated BioFET had a high selectivity (an eightfold signal difference) among the cDNA detections and a good sensitivity ($\approx 45$ $\mu$A at [nucleic acid] = 1 pm) among the viral RNA detections, respectively. The detection limit in the saliva samples is expected to pass beyond 1 fm which was the lowest concentration tested in this work, indicating the feasibility to detect viral RNA without PCR processing (i.e., biochemical amplification). To resolve the complicated pretreatments for clinical samples, we evaluated the sensor performance in the presence of a viral envelope lysis detergent (SDS) and a RNase-inactive reagent (DEPC). The matrix insensitivity was confirmed with the varying media, and the tests performed in SDS and DEPC had similar signal amplitudes compared to what were measured in diluted human saliva. Due to an ease of sample collection/pretreatment, the portable BioFET platform can carry out COVID-19 assay within 20 min (total turnaround: $\approx$1 h). Conclusively, this proposed system has a high potential for an on-site COVID-19 screening that can be widely deployed at frontline medical infrastructures, benefiting the disease control.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Data Availability Statement
The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Keywords
COVID-19, EDL-gated BioFET, portable biosensors, saliva-based diagnostics, SARS-CoV-2, viral RNA screening
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