Detection of SARS-CoV-2 with Solid-State CRISPR-Cas12a-Assisted Nanopores

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ABSTRACT: The outbreak of the SARS-CoV-2 caused the disease COVID-19 to spread globally. Specific and sensitive detection of SARS-CoV-2 facilitates early intervention and prevents the disease from spreading. Here, we present a solid-state CRISPR-Cas12a-assisted nanopore (SCAN) sensing strategy for the specific detection of SARS-CoV-2. We introduced a nanopore-sized counting method to measure the cleavage ratio of reporters, which is used as a criterion for positive/negative classification. A kinetic cleavage model was developed and validated to predict the reporter size distributions. The model revealed the trade-offs between sensitivity, turnaround time, and false-positive rate of the SARS-CoV-2 SCAN. With preamplification and a 30 min CRISPR Cas12a assay, we achieved excellent specificity against other common human coronaviruses and a limit of detection of 13.5 copies/μL (22.5 aM) of viral RNA at a confidence level of 95%. These results suggested that the SCAN could provide a rapid, sensitive, and specific analysis of SARS-CoV-2.

KEYWORDS: CRISPR, Cas12a, SARS-CoV-2, COVID-19, nanopore
method used an improved nanopore sized counting approach to examine the reporter size distributions and their relative abundance. We showed that the cleavage ratio of the intact circular ssDNA reporters could be quantified by the SCAN, which is used as a criterion for classifying the test as positive or negative. To guide the experiments, we developed a kinetic model to compute the reporter length distribution as a function of the cleavage reaction time. This experimentally validated model revealed the trade-offs between sensitivity, turnaround time, and false-positive rate of the SARS-CoV-2 SCAN. With a preamplification step and 30 min CRISPR Cas12a assay, we achieved a limit of detection (LoD) of 13.5 copies/μL (22.5 aM) of SARS-CoV-2 viral RNA at a confidence level of 95%. The SARS-CoV-2 SCAN has also shown excellent specificity against three other common human coronaviruses. Our results suggested that the SCAN could provide a rapid, sensitive, and specific analysis of SARS-CoV-2.

■ RESULTS AND DISCUSSION

Working Principle of Nanopore-Sized Counting. Figure 1a illustrates the working scheme of the SARS-CoV-2 SCAN using nanopore-sized counting. There were three streamlined steps: reverse transcription and amplification, Cas12 assay, and nanopore-based molecule classification and counting. In the first step, a one-step reverse-transcription polymerase chain reaction (RT-PCR) of SARS-CoV-2 RNA was conducted to improve the overall sensitivity of the system.31,32,34 After amplification, the complementary DNA (cDNA) amplicons were introduced to the sequence-specific CRISPR RNA (crRNA) and Cas12a ribonucleoprotein mixture (a.k.a., RNP). Upon specific cDNA binding, Cas12a could conduct collateral cleavage on the surrounding ssDNA reporters.39 We used circular M13mp18 single-stranded DNA (ssDNA) as the reporter in this study which is widely available and has an excellent signal-to-noise ratio in nanopore measurement. In the trans-cleavage process, the mother circular ssDNA reporters could be digested into daughter linear ssDNAs, and the daughter reports could be further digested into granddaughter reporters (positive case in Figure 1a). However, if SARS-CoV-2 viral RNAs were not present in the analyte solutions, then the Cas12a remains inactive and will not degrade the mother circular ssDNA reporter (negative case in Figure 1a).

These uncleaved mother reporters and multigenerational cleaved daughter reporters were then counted and classified by a glass nanopore sensor to infer their size and concentration distributions. Figure 1b shows two representative ionic current time traces for a positive and a negative sample (no target control), using a glass nanopore with a diameter size less than 10 nm (Figure S1a,b). For the positive sample (presence of SARS-CoV-2 RNA), it is apparent that molecular translocation events become more frequent but have less current...
blockage magnitude as compared to the negative sample. This is because the mother circular reporters were cleaved to many smaller daughter linear reporters. Our previous work analyzed the resulting reporter concentration by nanopore digital counting without taking the daughter reporter size distribution into consideration.\textsuperscript{30} This assumption is not exactly accurate if the reaction time is short. To further analyze the daughter reporter size distribution and its relative abundance, we here adopted a nanopore-sized counting method. First, the conventional event duration versus blockage was obtained from the ionic current time trace data (Figure S1b). As shown in Figure 1c, it is evident that the event duration and blockage in a positive case are smaller than in a negative case. Second, we classified each event based on its event charge deficit (ECD), which is used as the molecule size approximation.\textsuperscript{40} The ECD is defined as $ECD = \int_{\text{event}} \Delta I(t) \, dt \approx \Delta I \tau$,\textsuperscript{41} where $\Delta I$ and $\tau$ are the duration and blockage of each event, respectively. It was previously demonstrated that ECDs of DNA translocations with the same length are identical regardless of whether the molecules are in a linear, circular relaxed, or supercoiled form.\textsuperscript{40} An ECD bin size of 20 fC was used in this study unless otherwise stated. Third, the event rate of each ECD subpopulation was obtained by normalizing subpopulation event numbers by the nanopore reading time (Figure 1d). This enables us to quantify the reporter subpopulation concentration through $R_i = C_i \alpha N_A$, where $N_A$ is the Avogadro constant and $\alpha$ is usually referred to as the capture rate.\textsuperscript{42--45}

The mean ($\mu$) and standard deviation ($\sigma$) of the ECD value in the negative cases was used to establish an ECD threshold ($ECD_t = \mu - 2\sigma$, dashed line in Figure 1d). An event must have an ECD larger than $ECD_t$ to be classified as an intact mother reporter (i.e., to the right of the dashed line in Figure 1d). To quantify the percentage of the mother reporters being cleaved into daughter reporters, we defined the cleavage ratio (CR) as the ratio between the cleaved mother reporter ($C_0 - C_{\text{uncleaved}}$) to the total initial mother reporter ($C_0$). This cleavage ratio can be experimentally obtained by evaluating the aggregated event rate as

$$CR = 1 - \frac{\sum_j R_{pi}}{\sum_j R_{nj}},$$

in which the event rate summation is over all events with ECD larger than $ECD_t$ (thus representing the intact mother reporter concentration in the system) and $n$ and $p$ denote the negative and positive cases, respectively. The increase in the cleavage ratio of a testing sample would confirm the existence of the target viral RNA.

\textbf{SARS-CoV-2 Cas12a Assay Validation.} Prior to the Cas12a assay, one-step RT-PCR was conducted to increase the number of molecules and boost the signal. We utilized the primers designed by the United States Centers for Disease Control and Prevention (CDC) targeting the N2 region of the SARS-CoV-2.\textsuperscript{46} We conducted a real-time RT-PCR with $2 \times 10^5$ copies/$\mu$L SARS-CoV-2 viral RNAs for a duration of 45 cycles. The fluorescent signal confirmed the amplification after

![Figure 2.](image_url)
20 cycles (Figure S2). To further confirm the amplicon product, we conducted gel electrophoresis of the RT-PCR products, which showed a sharp band at 67 bp, as expected with our RT-PCT primer design (Figure 2a).

Afterward, we conducted the SARS-CoV-2 specific Cas12a assay with a reaction time ranging from 0 to 30 min. The reaction was stopped by adding the DNA gel loading dye (6X), which contained ethylenediaminetetraacetic acid (EDTA). Figure 2b presents the gel electrophoresis results of the Cas12a assay. We observed several important features. First, the mother reporter appeared in a double band around 7 kbp. This is due to the fact that electrophoretic mobility of DNA in gels could also be affected by the conformation of the DNA.47 Second, the daughter reporters become visible after 2 min, indicating the cleavage of mother reporters. Third, the primers were observed as a blurred short band in all cases. Fourth, as we increased the reaction time, more mother reporters were cleaved. At 30 min, the 7.2 kilonucleotides (knt) band of the uncleaved mother ssDNA become barely visible.

To examine the Cas12a cleavage kinetics at a much longer time scale, we conducted another test by intentionally extending the Cas12a reaction time up to 24 h. We found that all mother and prior generation daughter reporters were completely cleaved to be less than 250 nt after 24 h (Figure S3). This suggests that the trans-cleavage activities indiscriminately and continuously affect both the mother reporters and the partially cleaved daughter reporters. As a control, we also conducted the Cas12a assay for no target samples to confirm that no degradation of the mother reporters would occur in the absence of SARS-CoV-2 amplicons (Figure S4).

**Highly Sensitive Nanopore Measurement of the Cleavage Ratio.** After validating the Cas12a assay with gel electrophoresis, we set out to conduct SARS-CoV-2 detection with the glass nanopore. We conducted the Cas12a assay with different reaction times from 0 to 30 min and recorded the reporter translocations through the nanopore under 400 mV bias (Figure S5). In our glass nanopore measurement, DNAs with sizes less than 100 bp are often too small to be detected. Those significantly cleaved reporters with lengths less than 100 bp and RT-PCR amplicons (67 bp) could not contribute to the detected signals. In addition, in our previous study,48 we showed that the other components in the assay, such as Cas12a proteins, do not create signals in the nanopore experiment. Therefore, all the signals in the nanopore measurements are caused by the reporters with a length above the detectable threshold (a few hundred nucleotides).

Figure 2c shows the extracted translocation dwell time versus ionic current blockage at each reaction time. A clear shift of the blockage-duration distribution was observed when increasing the reaction time, indicating the changing populations of differently sized reporters. To quantify the abundance of differently sized reporters, we used an ECD bin size of 20 fC to classify the events into different subpopulations and calculated its corresponding event rate. Figure 2d shows the event rate distribution for all subpopulations. As shown, the event rate of larger ECDs (longer reporters) is reducing as the cleavage reaction goes, whereas the event rate of smaller ECDs (shorter reporters) is increasing. Since the concentration of the analyte could be quantified by the event rate in the nanopore experiment, these measurements give us the capability to quantify the relative abundance of differently sized reporters.

To quantify the cleavage ratio at different reaction times, we utilized the nanopore-sized counting method. The case at 0 min of reaction was considered as the negative case to establish the ECDt. The cleavage ratio (CR) at each reaction time was then obtained by using eq 1. As shown in Figure 2e, the CR was at 0.12 after 1 min of reaction and increased with increasing reaction time. For instance, CR was measured as 0.92 at 30 min of reaction. As the reaction time increases, more daughter and granddaughter reporters would be created, which increases the possibility of multturn cleavage. Therefore, the cleavage ratio increases at a lower rate as we increase the reaction time. It is notable that we were able to detect the cleavage activity after 1 min in nanopore reading, whereas no cleavage was barely visible in the gel after 1 min of reaction (Figure 2b). This indicates that the nanopore is a much more sensitive readout system for CR measurement.

**Cleavage Numerical Modeling and Validation.** In order to guide our experiments for rapid and sensitive detection of the cleavage ratio using nanopore experiment, we sought to develop a model to estimate the distribution of reporter length over the trans-cleavage process. At the start of the reaction, we assumed that each mother reporter has an identical length of $L_{m0}$. As the reaction starts, the reporters (including mother and daughter reporters) are randomly picked by the activated Cas12a. The probability that a reporter was picked and cleaved by the Cas12a was proportional to its cross-sectional area $R_g^2$ where $R_g$ is the gyration radius of the DNA coil. It has been shown that the gyration radius of DNA is proportional to the square root of its length ($L^{1/2}$).42 Therefore, the longer the reporter was, the more chance that it was bound to and cleaved by the Cas12a. The reporter was cut into two parts randomly. We assumed a normal distribution to model the cleavage position in a report. The velocity of the cleavage was modeled by \( v(t) = \frac{k_{cat}}{K_M + [S(t)]} \) in which $[E_0]$ is the initial concentration of enzyme (activated Cas12a), $[S(t)]$ is the substrate concentration (mother and daughter reporters), and $k_{cat}$ and $K_M$ are the catalytic rate and the Michaelis constant, respectively. We used previously reported $k_{cat}$ and $K_M$ values of 0.6 (1/s) and 2.7 $\times$ 10^{-6} M, respectively.49 This model was numerically implemented in a customized MATLAB code (see Figure S6 for model flowchart and results).

In order to validate this numerical model for predicting the reporter size distribution after reaction, we conducted the Cas12a assay at three enzyme concentrations (7.5, 15, and 30 nM) with reaction times ranging from 0 min to 24 h. Figure 3a presents the gel electrophoresis results. As expected, a higher enzyme concentration indeed results in faster cleavage activity since the cleavage velocity is proportional to the enzyme concentration. We extracted the length distribution of the reporter from the gel images by measuring the normalized grayscale values using ImageJ software.50,51 The normalized reporter length distributions were then overlaid with the results produced by our model. As shown in Figure 3b, shorter daughter reporters were produced at a fixed reaction time as enzyme concentration was increased. Also, both model and gel results showed that the mother reporters were cleaved entirely after 24 h. The distribution of reporter length captured by our model is consistent with the gel electrophoresis results, which validates our model.

**Trade-Off among Sensitivity, Reaction Time, and False-Positive Rate.** Since the cleavage ratio (CR) was used...
to distinguish a positive and a negative sample, we calculated the CR with the model-produced length distributions. The model-derived CR values were then compared with the nanopore-measured CR values using the sized counting method (eq 1). We measured the CR at different enzyme concentrations (7.5, 15, and 30 nM) with reaction times ranging from 0 to 30 min. As shown in Figure 4a, the model predicted CR values agree excellently with that measured by the nanopore.

With the capability to calculate the CR at varying activated Cas12a and reaction time, we were able to estimate the sensitivity and turnaround time of the SCAN system at any given CR threshold (CRt) for a positive call. Figure 4b presents the minimal required reaction time versus the activated Cas12a enzyme concentration. The activated Cas12a enzyme concentration is equal to the smaller values between SARS-Cov-2 amplicons and nonactivated Cas12a concentration in the system. As shown in Figure 4b, at any given CRt for a positive call, there is a trade-off between sensitivity (CRt) and false-positive rate (CR). With a fixed CRt, a higher CR (less false-positive) calls for a longer reaction time Tt. With a fixed CRt, reducing the CR (better sensitivity) also requires a longer reaction time (longer turnaround). By coupling with a preamplification step, the CR can be effectively enhanced and thus significantly reduce the required Tt (turnaround time).

Analytical Specificity and Sensitivity of SARS-CoV-2 SCAN. We then went on to evaluate the analytical sensitivity and specificity of SARS-CoV-2 SCAN. We used heat-inactivated SARS-CoV-2 RNA samples at different concentrations ranging from 2 to 200 copies/μL. In addition, three other human coronaviruses (229E, NL63, and OC43) with a concentration of 5 × 10⁵ copies/μL were used as the nontarget negative controls to evaluate the specificity. Each sample (5 μL) was first amplified by RT-PCR for 45 cycles (Figure S7). The product of the RT-PCR was added to 30 nM nonactivated Cas12a. The Cas12a cleavage assay was conducted at 37 °C for 30 min. Afterward, the nanopore-sized counting was conducted to determine the cleavage ratio. A positive/negative call was subsequently derived by comparing the obtained cleavage ratio with CRt (dashed line in Figure 5a). The CRt was defined in our experiment as μ₀ + 2σ₀ (0.089), where μ₀ and reaction time (Tt), CR = vTt. The cleavage velocity v is proportional to the activated Cas12a concentration Cenzyme as v = βCenzyme. Therefore, one can see that Cenzyme Tt/CR should equal to the constant coefficient β. This relationship suggests there is a trade-off between sensitivity (Cenzyme), reaction time (Tt), and false-positive rate (CR). With a fixed Cenzyme, a higher CR (less false-positive) calls for a longer reaction time Tt. With a fixed CRt, reducing the Cenzyme (better sensitivity) also requires a longer reaction time Tt (longer turnaround). By coupling with a preamplification step, the Cenzyme can be effectively enhanced and thus significantly reduce the required Tt (turnaround time).
and $\sigma_0$ (0.017) are the mean and standard deviation of the cleavage ratio obtained from no target controls (i.e., SARS-CoV-2 sample at zero concentrations). Figure 5a presents the cleavage ratios of all SARS-CoV-2 and nontarget human coronavirus samples. As shown, the cleavage ratios for all nontarget human coronavirus samples (circle, diamond, and star symbols in Figure 5a) are less than CR and were correctly classified as negatives. This confirmed that the SCAN sensor has an excellent specificity against SARS-CoV-2.

For the serially diluted SARS-CoV-2 samples (square symbols in Figure 5a), we found that concentrations higher than 15 copies/μL were classified correctly as positives since their cleavage ratios are much larger than CR. The high cleavage ratio in these samples indicates the majority of the mother reporters were cleaved after 30 min of reaction. However, we started to observe false negatives results in samples with concentrations lower than 15 copies/μL. For instance, 1 out of 5 samples at a concentration of 10 copies/μL and 4 out of 5 samples at 2 copies/μL were misclassified as negatives. To estimate the LoD of SARS-CoV-2 SCAN, we examined the hit rate at each different SARS-CoV-2 concentration. The hit rate is defined as the number of replicates with a detected outcome per the total number of replicates tested. As shown in Figure 5b, the hit rate started to roll off from 1 to 0.8 when the concentration decreased from 15 to 10 copies/μL. We fit the experimental hit rate data with a logistic curve (Figure 5b). On the basis of the fitting curve, we estimated the LoD of SARS-CoV-2 SCAN as 13.5 copies/μL (22.5 aM) at a 95% confidence level.

**CONCLUSIONS**

In summary, we introduced and evaluated a sized counting method for nanopores-assisted CRISPR-Cas12a-based detection of SARS-CoV-2. A sized counting scheme for cleavage quantification of the reporters in the Cas12a assay was introduced by utilizing ECD values from the nanopore experiment. We found that the nanopore is a sensitive readout system to measure the cleavage ratios, a criterion used for positive or negative classification. A kinetic cleavage model was developed and experimentally validated to predict the reporter length distribution. This model revealed the trade-offs between sensitivity, reaction time, and false-positive rate in the SARS-CoV-2 SCAN. These trade-offs could be relaxed by coupling with preamplification steps. With a 25 min RT-PCR step, 30 min of CRISPR Cas12a assay, and 10 min of nanopore reading (65 min of assay-to-result time), we achieved a limit of detection of 13.5 copies/μL (22.5 aM) of viral RNA. The SARS-CoV-2 SCAN showed excellent specificity with no cross-reactivity to other human coronaviruses. These results suggested that the solid-state CRISPR-Cas12a-assisted nanopores could provide a rapid, sensitive, and specific analysis of SARS-CoV-2.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at [https://pubs.acs.org/doi/10.1021/acs.nanolett.1c02974](https://pubs.acs.org/doi/10.1021/acs.nanolett.1c02974).

Methods section, including materials and chemicals, SARS-CoV-2 RT-PCR and Cas12a assay, glass nanopore fabrication and characterization, and nanopore sensing...
and data analysis sections. Nanopore characterization and current drop—dwell time measurements. Results of RT-qPCR. Gel electrophoresis results of the Cas12a assay products at five reaction times from 0.5 to 24 h. Gel electrophoresis results of the no target negative control samples. Translocation recording of ssDNA reporters at different reaction times through the glass nanopore. Algorithm and the model details for estimation of the reporter length distribution over the trans-cleavage process. RT-PCR results for the analytical specificity and sensitivity test (PDF)

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**Author Contributions**

W.G. conceived the concept and together with X.L.L. supervised the study. R.N. and Z.T. designed and conducted the RT-qPCR. R.N. and Y.J. developed and conducted the Cas12a assay. R.N. conducted the nanopore fabrication and sensing experiment. W.G. and R.N. cowrote the manuscript, with discussion from all authors.

**Notes**

The authors declare no competing financial interest.

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