Research paper

A CRISPR-Cas12a-based specific enhancer for more sensitive detection of SARS-CoV-2 infection

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Abstract

Background: Real-time reverse transcription-PCR (rRT-PCR) has been the most effective and widely implemented diagnostic technology since the beginning of the COVID-19 pandemic. However, fuzzy rRT-PCR readouts with high Ct values are frequently encountered, resulting in uncertainty in diagnosis.

Methods: A Specific Enhancer for PCR-amplified Nucleic Acid (SENA) was developed based on the Cas12a transcleavage activity, which is specifically triggered by the rRT-PCR amplicons of the SARS-CoV-2 Orf1ab (O) and N fragments. SENA was first characterized to determine its sensitivity and specificity, using a systematic titration experiment with pure SARS-CoV-2 RNA standards, and was then verified in several hospitals, employing a couple of commercial rRT-PCR kits and testing various clinical specimens under different scenarios.

Findings: The ratio (10 min/5 min) of fluorescence change (FC) with mixed SENA reaction (mixed-FCratio) was defined for quantitative analysis of target O and N genes, and the Limit of Detection (LoD) of mix-FCratio with

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1. Introduction

Since December 2019, the outbreak of COVID-19, caused by the infection of the SARS-CoV-2, has rapidly spread throughout the world, and is now a global pandemic [1]. Till June 1, 2020, the outbreak has affected 216 countries, areas and territories, infected 6 million people, and caused more than 370 thousand of death [2]. One of the greatest public health concerns in combating the pandemic is a prompt response to the urgent demand for rapid and accurate diagnosis of the virus. Currently, nucleic acid amplification-based molecular diagnostics (MDx) is the most accurate, fast and affordable and thus the preferred method for diagnosis of SARS-CoV-2 infection, and the real-time reverse transcription PCR (rRT-PCR) kits have been successfully developed by quite a few laboratories and commercial companies [3]. However, since its clinical application at least six months ago in China, the diagnostic performance of rRT-PCR for SARS-CoV-2 has brought some urgent challenges, particularly the uncertain negative or positive readouts associated with the frequently encountered high Ct-value “grey zones” [4–8]. Besides of “human error” factors such as misconducted sampling, unqualified reagents and uncalibrated diagnostic equipment, inefficient RT reaction and PCR amplification of clinical samples with very low viral loads are likely the major intrinsic causative factors for the fuzzy rRT-PCR readouts and uncertain diagnosis. Although repetitive sampling and assays are implemented for final confirmation of the diagnosis, these “trouble shooting” efforts are time-consuming and may still fail to detect the low viral load samples from some mild or asymptomatic patients, or from the recovering patients, resulting in false-negative diagnosis that may cause serious public concerns in battling against the pandemic (Fig. 1).

With the characterization of non-specific trans-cleavage activities against single-stranded nucleic acids in several CRISPR-associated (Cas) proteins, e.g., Cas12, Cas13 and Cas14 [9–15], Clustered Regularly Interspaced Short Palindromic Repeats Diagnostics (CRISPR-Dx) technology [16, 17] was established and has been developing rapidly. The underline mechanism for CRISPR-Dx, as illustrated by the Cas12a-based HOLMES system for example [18], is based on the efficient trans-cleavage activity against a fluorophore quencher (FQ)-labeled single-stranded DNA reporter by Cas12a triggered upon target DNA recognition, which is guided by a specific CRISPR RNA (crRNA), generating exponentially increasing fluorescence signal within several minutes. With this mechanism, here, we design a Specific Enhancer for detection of PCR-amplified Nucleic Acids (SENA) to improve both the detection sensitivity and the specificity of rRT-PCR, solving the uncertainty problem in COVID-19 diagnosis and thus providing a simple and low-cost companion diagnosis for combating the pandemic.

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1.1. Research in context

Evidence before this study

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has exploded all over the world and is already a global pandemic. rRT-PCR technique is the current gold standard for molecular diagnosis of COVID-19 and is recommended by both the Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO). However, the diagnostic performance of rRT-PCR for SARS-CoV-2 has been challenged by the frequently encountered “grey zone” problem that is caused by high Ct values. Usually, sample re-collection and retesting are required, which is time-consuming and labor-intensive, and a simple and low-cost technology for solving this “grey zone” problem and correcting false positive or false negative diagnosis is thus urgently needed.

Added value of this study

We here created a CRISPR-based diagnostic technology, namely the Specific Enhancer for PCR-amplified Nucleic Acid (SENA), and proved that the LoD per reaction of SENA is at least two copies less than that of rRT-PCR. Besides, the mix-FCratio was defined for quantitative analysis of SENA readouts and the cut-off values for mix-FCratio were also determined. Then, SENA was verified with 295 clinical specimens with 4 false negative and 2 false positive rRT-PCR diagnosis identified. Moreover, SENA also resolved the uncertainty problems of 21 specimens that fell in the rRT-PCR “grey zone”.

Implications of all the available evidence

SENA is a safe, simple, stable, quick and low-cost diagnostic tool with no need of extra instruments. More importantly, it may efficiently eliminate the uncertainty problems of PCR-based diagnosis and thus, may have great potential in applications such as COVID-19 diagnosis and many other clinical scenarios.
Fig. 1. Schematic description of SENA and its application as a confirmation diagnosis for rRT-PCR diagnosis of COVID-19. Generally, nucleic acids are extracted from the clinical specimens such as pharyngeal swabs of the suspects of SARS-CoV-2 infection and then subject to rRT-PCR analysis. The diagnostic reports are based on the Ct cut-off values guided by the supplier of rRT-PCR kits. However, high Ct-value designated “grey zone” associated uncertain fuzzy readouts are often encountered. Besides, some probably false-positive or false-negative cases may be indicated by their atypical clinical symptoms or signs. For all these cases, the corresponding rRT-PCR products can be sent to another physically isolated room for SENA analysis and the ambiguity may be clarified by SENA with its positive and negative cut-off mix-FCratio. The real-life data related to these scenarios revealed in this study are shown in the figure and details are illustrated in the text. RJ, JNCDC and SZII are the names of the hospitals and the number indicates the overall number of patients identified. While P140 was a patient in DF hospital, and two distinct samples from P140 were identified to be false-negative. For details, please refer to Supplementary Table 3.
rRT-PCR kit supplier, Supplementary Table 1b), 1 μM FQ-reporter,
and 1 U/μl RNase inhibitor (TaKaRa).

2.2.2. SENA detection
To avoid the aerosol contamination of the MDx laboratory, after the
rRT-PCR reaction, their products must be transferred to a physically
isolated room to perform SENA detection. It is also important to
choose proper SENA detection reagents corresponding to the rRT-
PCR kits. To prepare a 20 μl SENA reaction system, with correspond-
ing positive and negative controls, 2 μl PCR products and 8 μl
RNase-free H2O were mixed with 10 μl 2 × SENA reagent, and the
mixture was then measured on an appropriate fluorescence reader
with FAM fluorescence collected following the programs: 48 °C 30 s
per cycle, 20 cycles. Both the slope and the Fluorescence Change (FC)
can be calculated at any time points as desired.

2.3. Next generation sequencing (NGS)

The rRT-PCR products were purified by AMPure XP beads (Beck-
man Coulter Life Sciences, US), and libraries were then constructed
following the procedures of end repair, dA-tailing and adaptor liga-
tion, with the StepWise DNA Lib Prep Kit for Illumina (ABclonal,
China). After PCR amplification, samples were sequenced on Illumina
Miniseq to produce 2 × 150 bp paired-end reads. After adaptor trim-
ming and quality trimming, the clean reads were mapped to the ref-
rence genome of SARS-CoV-2 (MN908947.3) using Bowtie2 [19].

2.4. Systematic titration and regression analyses

2.4.1. Systematic titration experimentation

2.4.1.1. The standard RNA templated. The SARS-CoV-2 RNA standards
were purchased from Genewell (Shenzhen, China). According to the
supplier’s information, three plasmids containing the whole sequen-
ces of N and E genes, and partial sequence of the Orf1b, i.e.,
from 13,237 to 13,737 of the SARS-CoV-2 complete genome
(MN908947.3), were transcribed in vitro individually. The RNA prod-
ucts were mixed with equal molar, aliquoted with addition of 1 μg of
human RNA per tube, and subject to lyophilization and subsequent
quantification with digital PCR. This SARS-CoV-2 RNA standard dry
powder containing 1808 copies of O gene, 1795 copies of N gene and
1160 copies of E gene was dissolved with 10 μl RNase-free water to
obtain the original stock solution (estimated 180.8 copies/μl of O,
179.5 copies/μl of N and 116 copies/μl of E).

2.4.1.2. The preparation of the serially diluted RNA templates. Pharyng-
eal swab samples were collected from 40 adult patients in Shenzhen
People’s Hospital by the Clinical Diagnosis Laboratory and the
nucleic acids of each sample were extracted with the pre-packaged
nucleic acid extraction kit (Da’An Gene, Ltd., Guangzhou, China),
according to the manufacturer’s instructions, ended up with 55 μl
extracts per sample. After rRT-PCR assays employing 5 μl of the
extracts from each sample, all of the samples were shown to be
SARS-CoV-2 negative. The remaining 50 μl extracts of each sample
were mixed together and 5 μl of the mixture was once again ana-
lyzed by rRT-PCR and confirmed to be SARS-CoV-2 negative. Then,
the mixed nucleic acid extract was used as the dilution buffer (totally
about 2 ml) for serial dilution of the SARS-CoV-2 RNA standard
stocks, generating desired concentrations (i.e., 5, 2, 1, 0.8, 0.6, 0.4, 0.2,
0.1, 0.05, 0.025, 0.01, 0.005 copies/μl), and 5 μl of each of the diluted
solutions were used as templates for rRT-PCR analysis, forming grad-
ient template concentrations (i.e., 25, 10, 5, 4, 3, 2, 1, 0.5, 0.25, 0.125,
0.05, 0.025 copies/Rx).

Replica setting: We analyzed 9 replicates for each of the concentra-
tions of 1 and 0.5 RNA template copies/Rx while 6 replicates for each of
the rest concentrations (Supplementary Fig. 1). rRT-PCR reactions:

rRT-PCR reaction kit was supplied by BG (Supplementary Table 1a),
who follows the primer sets recommended by Chinese CDC. Instead of
the recommended 40 cycles of PCR amplification, we set 45 cycles as
routine aiming at recording the maximum exact Ct values if possible.

SENA detection: Every amplicon of the rRT-PCR reactions was
subjected to SENA detection. Specially, for this experimentation, three sets of SENA reagents were individually used, O-SENA contains
the crRNA targeting the O sequence, N-SENA contains the N-targeting
crRNA, and the mix-SENA contains crRNAs for both sequences.

2.4.2. Choice of FC ratio as the standard readout for SENA detection and
mix-SENA as the standard reagent for clinical application

Three readout parameters were compared as shown in Supple-
mentary Fig. 1 (original data in Supplementary Table 2). The slope
(increase of fluorescence/min) represents the reaction rate of Cas12a
trans-cleavage activity, but in this experiment, it represents neither
the initial rate of the enzyme under limited substrate condition nor
the pure first-order reaction rate varies according to the substrate
concentration, particularly, demonstrated in cases of high template
concentration, in which, the slope goes down along with the increase
of the template concentration. In addition, when the substrate con-
centration is low, the slope of SENA is hard to be distinguished from
that of the negative control, ending with ambiguous cut-offs. The FC
(the fold of change of fluorescence between that of the sample over
that of the negative control at certain time point, usually 5–30 min)
does show clear differences between the positive amplicoms from that
of negative control, and it also shows certain quantitation character
particularly at the low concentration templates cases. Because of these
properties, FC has been a parameter used by a few users of CRISPR-Dx
[20]. However, it seems that the absolute value of the FC usually varies
along the reaction time and sometimes it is influenced by the change
of the fluorescence signal of the negative control. Besides, it is difficult
to determine the “best choice” of the FC recorded at certain time
points, which may cause confusing in clinical applications. It is clear,
we need a stable readout which reflects the dynamic process and the
quantitative correlation of SENA reaction with the low concentration
of the templates on one hand and should be robust and accurate for
clinical diagnosis on the other hand. We defined FC ratio, which is the
ratio between FCs of SENA detection at 10 min versus 5 min after
the beginning of the fluorescence reading. It not only measures the fluo-
rescence change of SENA against the negative control background so
that the quantity of the amplicoms, particularly at the low concentra-
tion range may be represented, but also normalizes the slope of the
fluorescence change of SENA to eliminate the complex background
differences. As shown in Supplementary Fig. 2, FC ratio significantly
amplified the positive signals and represents the quantitation of the
amplicoms at low template concentrations to certain extent.

As shown in Supplementary Fig. 2, the capacity of the three SENA
reactions are compared. It is obvious that N-SENA is the least sensitive
one, while although O-SENA seems much more sensitive than that of
N-SENA and largely comparable to that of mix-SENA, its signal at the
very low template concentration range seems uncertain in some
cases. Therefore, for clinical application, mix-SENA is the best choice.

2.4.3. Quality analyses of the rRT-PCR titration data
The quality of the Ct values vs the concentration of the standard
templates for rRT-PCR of the systematic titration experiment were
analyzed both empirically and statistically. Firstly, as shown in Supple-
mentary Table 2 and Supplementary Fig. 1, the amplification effi-
ciencies of the two genes represented by the valuable Ct readouts
were different. The apparently lower sensitivity of the N gene ampli-
fication is in contrast with the clinical experiences and might due to
the difference in the property of the templates used in different
experiments (laboratory standard RNA template vs clinical real viral
template). Secondly, although linear regression can be readily made
between the Ct values and the log2 (conc) (Supplementary Fig. 3a) as
that of the previously published tests, the quality of the regression as judged by the R²’s (Supplementary Fig. 3a) and the residues (Supplementary Fig. 3b) are clearly suboptimal likely due to the limited number of replicates in the experiment. On the other hand, this titration was designed with taking at least the two most fundamental limitation factors about the sensitivity of COVID-19 rRT-PCR diagnosis into consideration, i.e., the sampling ambiguity and the influence of the biological/chemical contaminants from the clinical samples. Therefore, the data will be used for determination of the LoD for rRT-PCR and SENA (Methods 4.4 and Supplementary Fig. 2), and the regression function will be used to estimate the “apparent Ct” values of the samples with Ct values greater than 45 (no amplification signal) but their SENA detection is positive (Methods 4.5 and Supplementary Fig. 4).

2.4.4. Determination of limit of detection (LoD) for rRT-PCR and SENA

The LoD values for rRT-PCR (N-Ct and O-Ct) and SENA (N-FCratio, O-FCratio and mix-FCratio) were estimated based on the systematic titration employing standard RNA templates (Supplementary Fig. 1 and Supplementary Table 2). The fractions of positive replicates versus the number of target molecules (copies) per reaction for N and O gene of COVID-19 were plotted and used the sigmoidal functions (1) to fit the data via R (software version 3.5.0). The 95% confidence intervals were derived by bootstrapping the model residues and were visualized by R (software version 3.5.0) with built-in ggplot2 library [21].

2.4.5. Regression of rRT-PCR Ct values and the SENA FCratio versus the concentration of the templates employing the data from the systematic titration

2.4.5.1. Regression of rRT-PCR Ct values with the concentration of the templates (copies/Rx). Since the PCR product increased exponentially with the initial concentration of the sample (x), and the Ct value of rRT-PCR parameter (y) was inversely correlated with the initial concentration, especially in the range of low copy number (low template concentration) samples, the power function equation (a**x) should be suitable for the data fitting. However, some of the experimental groups included very low initial sample concentrations (<1 copy/Rx), those amplification efficiencies should be different (particularly affected by sampling ambiguity) from that of the groups with high initial template concentration. Therefore, the power function formula with four parameters (Y=aX**b+eX**m) was used to match all the experimental group data to obtain a more accurate data model (Functions 1, Supplementary Fig. 4).

2.4.5.2. Regression of SENA FCratio with the concentration of the templates (copies/Rx). The exponential function (first order association kinetics of the interaction between a substrate and an enzyme, Y=a+b (1-e**X)) is used to fit the data of FCratio against the concentration of the templates. At low concentration (especially when the concentration is less than 2 copies/Rx), the FCratio is positively correlated with the template concentrations. However, when the template concentration reaches to 2 copies/Rx and more, the FCratio does not increase accordingly and the curve tend to be flattened out. In addition, as FCratio has already been normalized by the fluorescence signal of the negative background, it is stable and, in this case, we give the parameter Y₀ being set as a constant value between 0.9 to 1 (Functions 2 in Supplementary Fig. 5).

2.4.5.3. Regression of rRT-PCR Ct values with the SENA mix-FCratio values. In practice, quite significant portions of the clinical positive samples detected by mix-SENA with their FCratio readings higher than the positive cut-off, but with a negative PCR Ct value (40 - 45, depending on the scenario). Under certain circumstances, people may be interested to learn the copy number of the templates for the corresponding rRT-PCR assays or even the “probable” Ct values of these assays. With the aid of the above-mentioned two regression functions (1 in Supplementary Fig. 4 and 2 in Supplementary Fig. 5), these data could be estimated. One may firstly substitute the Y in Functions 2 by the measured FCratio value and the corresponding X can be calculated representing the “estimated concentration of the template”. Then this X value can be used to estimate the corresponding “estimated Ct-value” as the Y of Functions 1. We estimated all the ambiguous Ct values of the positive amplicons and plotted them against their corresponding mix-FCratio values (Fig. 4). All the real and estimated Ct values for both N and O genes are plotted against the corresponding FCratio values of mix-SENA as X axis. An exponential decay function (with X₀ = 1; When X<X₀, Ct=∞; otherwise, one phase decay) fits well to all the data (R²=0.9238) and is used for analyzing the clinical data and adjust the cut-off values accordingly (Fig. 4).

2.5. SENA detection of clinical samples

Nucleic acids are extracted from the total 295 clinical specimens, such as the pharyngeal swabs and nasopharyngeal swabs of the suspected of SARS-CoV-2 infection, which collected in Ruijin Hospital, Shenzhen Second People’s Hospital, and Jinan Center for Disease Control and Prevention, and then subject to rRT-PCR analysis. rRT-PCR reactions were conducted in a 25 μl reaction mixture following the instructions offered by the commercial suppliers of the reaction kits (refer to Supplementary Table 1a). Then, the corresponding rRT-PCR products were sent to another physically isolated room and 2 μl PCR products were carefully transferred to new tubes for SENA analysis (refer to Methods 2.2) and NGS (refer to Methods 2.3).

2.6. Detection of antibodies

The detection of anti-SARS-CoV2 antibodies was executed by the point-of-care microfluidic platform integrating a home-made fluorescence detection analyzer (Suxin, Shanghai, China). A total of 10 μl plasma was added into the loading chamber of microchip followed by the addition of 70 μl sample dilution buffer. After incubation for 15 min at room temperature, the microchips were loaded onto the fluorescence detection analyzer, and fluorescence signal was detected from the analyzer, following the manufacturer’s instruction.

2.7. Ethics statement

All experiments using human material were performed in accordance with the instructional guidelines and agreement of the Ethical Committee of Ruijin Hospital (#2018-48), and Shenzhen Second People’s Hospital (#202003009005). Written informed consent was waived given the context of emerging infectious diseases.

3. Results

3.1. Development and characterization of SENA

To prepare appropriate crRNAs for SENA detection, we firstly determined the amplicon sequences from several commercial rRT-PCR kits used in China and then designed specific crRNAs corresponding to each of the distinct amplicons (Supplementary Table 1a). Candidate crRNAs were prepared and analyzed individually in a SENA system, which comprised of, in addition to the crRNAs, Cas12a, FQ-reporter and the rRT-PCR products using templates of either the positive or negative controls. The apparently most appropriate crRNAs, i.e., the lowest fluorescence with the negative control and highest with the positive control, were chosen for the final formulation of the SENA assay reagents (data not shown). In general, four formulations of regents were prepared employing crRNAs against corresponding
The performance of SENA was quantitatively characterized via a systematic titration upon rRT-PCR amplicons employing pure SARS-CoV-2 RNA standards comprised of the O and N fragments, individually or mixed, as the templates. As it is aware that the viral nucleic acids extracted from patients’ samples such as nasopharyngeal swabs usually contain some biological and chemical contaminants that might inhibit the enzyme activities for reverse transcription and PCR reactions and is likely one of the causal effects attributed to the low efficiency of rRT-PCR in clinical analysis [22]. In order to mimic the clinical sampling for the titration experimentation, the RNA standards were serially diluted in buffer prepared by mixing the nucleic acid extracts from 40 COVID-19 negative people, generating RNA templates ranging from 0.025 to 25 copies per reaction (Rx).

Due to the Poisson distribution property of sampling, replica variations become extremely significant when the template copies in individual reaction are designed to be low, i.e., less than 3–4 copies/Rx, near the limit of detection (LoD) for rRT-PCR [21, 23], and extremely low, i.e., equal to and less than 1 copy/Rx. To overcome this sampling ambiguity problem, we performed 9 replicas for groups with 1 and 0.5 RNA template copies/Rx while 6 replicas for each of the rest concentrations. In addition, although the rRT-PCR assay supplier, BioGerm (BG, Shanghai, China), who follows the Chinese CDC recommended primer sets (Supplementary Table 1a), recommends 40 cycles of PCR amplification, we set 45 cycles as routine aiming at recording maximum exact Ct values if possible. After rRT-PCR reaction, all amplicons were subjected to 3 individual SENA reactions, i.e., O-SENA, N-SENA and mix-SENA with crRNAs targeting O gene, N gene and both, respectively.

Consistent with the theoretical analysis [23] and the rigorous experimentation [21], along with the decrease of the RNA templates to less than 3 copies/Rx, the rRT-PCR Ct values in some replicas, primarily that corresponding to the N gene, passed 38 (the cut-off for positive as recommended by the rRT-PCR kit suppliers) but were less than 40, which should be considered as entering the “grey zone”. The Ct values increased steadily when the concentration of the RNA templates further decreased, with more and more replicas showing one or both Ct values entering the “grey zone” and eventually all became “negative”, i.e., greater than 40 or even 45 (Fig. 2a, Supplementary Fig. 1). Employing Ct>38 as the cut-off for “positive” detection, we estimated the LoD for O and N genes with 95% confidence interval (CI) of this set of rRT-PCR assay as 3.3<4.0<6.1 and 4.0<4.1<4.4, respectively (Supplementary Fig. 2).

Most likely due to the influence of the complex combination of the targeted viral genomic fragments and the clinical sampling background, the LoD determined in this study was clearly higher than the published value of 2.0<2.5<3.7, which analyzed single target in a pure system [21].

The rRT-PCR amplicons were further analyzed by SENA detection with the measurement of the fluorescence signals for each corresponding replica. After comparison of the parameters of slope (increase of fluorescence/min) versus FC (the fold of change of fluorescence reading), we defined a parameter, FCratio, which is the ratio of the FC at 10 min to that at 5 min after the initiation of fluorescence reading (Supplementary Fig. 1 and Supplementary Table 2). We also found that in the cases with low concentrations of templates, the rRT-PCR efficiency of the two target genes (i.e., O and N) were different so as the SENA detection (Supplementary Figs. 1 and 2). In order to verify the existence of specific amplicons of SARS-CoV-2 nucleic acids in an individual rRT-PCR reaction, all of the amplicons of the replicas with RNA templates ranging from 0.125 to 2 copies/Rx were subjected to next generation sequencing (NGS) analysis. The results were found to be completely consistent with the perspective results of both O-SENA and mix-SENA. In addition, with mix-SENA, not only the signals are generally more significant than that of the O-SENA detection but also may resolve some of the ambiguity readouts found with N-SENA (Supplementary Fig. 1 and Supplementary Table 2).

Based on these results, the mix-FCratio was demonstrated as the most sure-proof index for rRT-PCR confirmation, and we empirically estimated that mix-FCratio≤1.145 for positive cut-off, and mix-FCratio≥1.020 for negative cut-off (Fig. 2a). Of course, these two parameters are subject to further verification and adjustment along with the increase of tested samples. Because SENA is rRT-PCR based, the same methodology for determining the rRT-PCR LoD was used to estimate that of SENA by this set of data, corresponding to both individual O and N fragments (Supplementary Fig. 2) and in combination as indicated by the mix-SENA (Fig. 2b). As expected, the N-SENA LoD...
(3.7 < \text{Ct} < 4.8 with 95% CI) is very close to that of the N-Ct of rRT-PCR, while the LoD of O-SENA (1.1 < \text{Ct} < 1.7 with 95% CI) is significantly lower than that of O-Ct (Supplementary Fig. 2). Although the LoD of mix-SENA (1.2 < \text{Ct} < 2.1 with 95% CI) is slightly higher than that of O-SENA (Fig. 2b and Supplementary Fig. 2), it is apparently caused by its capable of confirming some of the ambiguous amplicons in the extremely low concentration cases (Supplementary Fig. 2) and thus, mix-FCratio is chosen for clinical applications.

3.2. Verification of SENA in different clinical scenarios

SENA was further verified in a few hospitals, testing various clinical specimens and samples under different scenarios (Fig. 1) and employing fewer more commercial rRT-PCR diagnosis kits in addition to BG which was used in the titration experiment (Supplementary Table 1a). Totally 295 clinical samples or specimens (mainly pharyngeal swabs) collected from 282 individuals were tested by rRT-PCR followed by SENA detection (Supplementary Table 3). Except for asymptomatic carriers, all the cases of uncertain analytic and false positive or negative readouts of rRT-PCR diagnosis were encountered and finally confirmed or corrected by SENA detection.

Specifically, samples from 139 patients of Ruijin Hospital (RJ, Shanghai, China) were assayed by rRT-PCR employing diagnostic kits of LifeRiver (LR) and Beijing Genomics Institute (BGI), 137 of which had consistent readouts by all those of rRT-PCR kits, indicating two positive, 123 negative and 12 suspected that fell in the “grey zone” (Supplementary Table 3). SENA detection of these samples revealed not only the 12 suspected as negative but also identified one more positive among the original 123 negative individuals, clearly a case of false negative diagnosis (Supplementary Table 3). Besides, distinct rRT-PCR assay results, positive by BGI but negative by LR were shown for samples collected from 2 close contacts of COVID-19 patients and apparently asymptomatic (ref to Supplementary Table 3). However, the amplicons of both LR and BGI were shown as negative via SENA detection. All these ambiguous rRT-PCR amplicons (17 samples, ref to Supplementary Table 3) were finally analyzed by NGS, and the results were consistent with the SENA. Noticeably, the rRT-PCR false-negative COVID-19 patient was symptomatically mild at the point of admission with all the clinical laboratory tests negative but turned positive after 24 hours. On the other hand, although those 12 suspected patients had respiratory infection symptoms, they were finally excluded from COVID-19 according to the latest guideline for diagnosis and treatment from China National Health commission (the 6th edition). Similarly, in Shenzhen Second People’s Hospital (SZII, Shenzhen, China), 5 uncertain rRT-PCR readouts for 0 gene were found among 139 individuals. Three of them had Ct value of 39.47, 39.7 and 40.56, respectively but the following SENA detection gave mix-FCratio values less than 1.0 for all of them, indicating all negative. The other two individuals had Ct values of 38.87 and 39.22, while their mix-FCratio values were 1.581 and 1609, respectively, indicating positive for both. In addition, there were another three individuals with Ct values larger than 40 for 0 gene and 36.09, 35.88 and 37.98 for N gene, respectively; however, the following SENA detection showed mix-FCratio values were 1.39, 1.55 and 1.21, respectively, indicating all positive. All these amplicons were further confirmed by NGS analysis (Supplementary Table 3), obtaining consistent results with those of SENA. Consistently, the three SENA-negative individuals were finally excluded from SARS-CoV-2 infection after being rechecked by rRT-PCR after 24 hours (Supplementary Table 3). Based on above data, it is clear, SARS-CoV-2 infection suspects with either rRT-PCR Ct values falling in the “grey zone” or with clear patient-contact epidemiological history but negative rRT-PCR tests, are strongly recommended to perform SENA detection to minimize the possibility of misdiagnosis. On the other hand, in case an rRT-PCR-positive suspect does not demonstrate any COVID-19 clinical symptoms and/or signs, SENA detection is also strongly recommended to eliminate either false-positive diagnosis or misdiagnosis of the so-called “asymptomatic carrier” or “asymptomatic patient”.

Besides of preventing false-negative or false-positive diagnosis, the highly sensitive property of SENA may also assist in providing evidence of viral clearance for COVID-19 recovering patients. A female patient in Dongfang Hospital (DF, Shanghai, China) was confirmed as COVID-19 positive by both rRT-PCR and CT scanning and showed ground-glass opacities mixed with consolidation along the subpleural area (Fig. 3). Accordingly, the SENA test was positive with the mix-FCratio of 1.43. After the hospitalization, the patient was further analyzed by rRT-PCR at two time points, obtaining all negative results with bilateral nasal and pharyngeal swab specimens. However, the mix-FCratios of SENA for some of her specimens were 1.64, 1.36 and 1.00, respectively, indicating that the virus was contained and yet to be cleared. On the seventh day, both rRT-PCR and the corresponding SENAs detection for all of her specimens were negative and these results were confirmed by NGS and consistent with her normal CT scanning results (Fig. 3). Thus, she was discharged from the hospital and safely back to home. Similar cases were found in Jinan of Shandong Province, China, where the nasal samples from two recovering COVID-19 patients were tested negative by rRT-PCR but clearly positive by SENA (Supplementary Table 3). Considering a certain percentage of the recovered patients discharged from hospitals were reported to be re-detectable positive (RP) [24], the incomplete clearance of the SARS-CoV-2 virus ahead of discharge might be one of the possible causes. Therefore, it could be necessary to consider more sensitive detection approaches such as SENA as a potential index of viral clearance.

To reconfirm and/or improve the cut-off values for SENA mix-FCratio, the ambiguous Ct values were re-estimated using the regression functions derived from the rRT-PCR assays with titrated standard RNA templates (Supplementary Figs. 4 and 5), and then the Ct values (both estimated and detected) were plotted against the corresponding mix-FCratios (Fig. 4). Combining the data from both RNA standards and clinical samples, it is clear that SENA detection is of both high sensitivity, identifying real positive samples with Ct values as high as more than 43 (approaching 50 as estimated), and high specificity, identifying real negative samples with Ct values as low as 39. Therefore, SENA can effectively eliminate uncertain diagnosis of rRT-PCR assays for SARS-CoV-2 infection. In addition, the cut-off value for SENA mix-FCratio remains unchanged as 1.145 for positive diagnosis while slightly increased to 1.068 for negative (Fig. 4), which is supposed to further increase along with the clinical applications.

4. Discussion

Instead of developing a closed CRISPR-Dx system, which ideally should be comprised of both target nucleic acids amplification and CRISPR-Cas-based trans-cleavage assays, SENA was created here to match the commercially available and widely applied rRT-PCR kits, and to solve the uncertainty challenge of the rRT-PCR “grey zone” in COVID-19 diagnosis. As expected, SENA specifically increases both the detection sensitivity (i.e., with the LoD of 1.6) and the specificity (i.e., with false positive and false negative samples detected) in COVID-19 diagnosis.

In a SENA detection system, the amplicons of rRT-PCR are taken as the target nucleic acids for the Cas12a-based trans-cleavage reaction, and the remaining single-stranded PCR probes that are fluorescently labelled may have influence on the SENA readouts. Firstly, the crRNAs were carefully designed to avoid targeting either the primers or the probes. Therefore, the remaining the probes will not trigger the trans-cleavage activities of Cas12a. Secondly, as only a small amount of the amplicons is added in the SENA reaction system, the remaining fluorophore will have no influence on the SENA results even if the rRT-PCR system uses the same fluorophore as that in SENA. On one
hand, the addition of cleaved rRT-PCR probe increases only the background signal of SENA other than the $\text{FCratio}$. On the other hand, the un-cleaved rRT-PCR probe is also the target for Cas12a trans-cleavage, which is only $1/25$ of the FQ-reporter in amount and will increase the saturation signal instead of the $\text{FCratio}$. Taken together, one may conclude that the remaining rRT-PCR probes have no influence on the SENA results. Considering the fact that detection of 5(6)-carboxyfluorescein (FAM) with its maximum wavelength of excitation and emission at 494 nm and 522 nm, respectively, is supported by most fluorophores as previously reported [18].

Since the outbreak of COVID-19 pandemic, dozens of rRT-PCR diagnosis kits that use distinct PCR primer pairs and probes have been successfully developed and are commercially available. Here in this study, both LoD and cut-off values were determined for SENA with the employment of one of them, which is also commercially available in China. Although the LoD of SENA can be different for distinct rRT-PCR kits, the SENA cut-off values were shown to be consistent among the three tested rRT-PCR kits in this work (ref to Fig. 4 and Supplementary Table 3). For example, the cut-off values were empirically estimated as mix-$\text{FCratio} \geq 1.145$ for positive and mix-$\text{FCratio} < 1.020$ for negative with the employment of standard samples. Then, after the verification of SENA detection with 295 clinical specimens, using three different rRT-PCR kits (Supplementary Table 3), the cut-off values for positive remained unchanged and the cut-off value for negative slightly changed from 1.020 to 1.068. Therefore, when a mix-$\text{FCratio}$ falls between 1.068 and 1.145, which is the “grey zone” of SENA detection at present, either NGS or clinical symptoms should be used to help diagnosis. Also, it is highly recommended that following researchers may precisely determine the LoD for a specific rRT-PCR kit as well as the cut-off values in future. With the increase of the samples tested, the SENA cut-off value for negative may become slightly larger, generating a smaller “grey zone” and consequently leading to less uncertainty in SENA diagnosis.

Besides, to precisely calculate the LoD of SENA, the quality of the rRT-PCR data in this study was also carefully evaluated before further SENA analysis. In general, most measuring techniques generate a signal response that is proportional to the amount of measurable present. For example, measured absorption is proportional to the concentration of the dissolved measurand as predicted by the Beer-Lambert law [25]. Regarding to rRT-PCR, the measured Ct values are logarithmic responses proportional to the log base 2 ($\log_2$) of the concentration of the measurand (the number of target molecules present). We thus plotted the Ct values against the $\log_2$ of their corresponding template concentrations, in which the Ct value decreased with the increase of the template concentration. Although the few most diluted samples were outside the linear range of rRT-PCR standard curve, which might be due to sampling background noise and limited number of replicates in the experiment (Supplementary Fig. 3), the data were on the whole fitted to a straight line. On the other hand, while linear data generated by linear measurements are normally distributed in a linear scale with the measurand concentration changing [26], quantitative real-time PCR data show normal distribution in a logarithmic scale [27]. To confirm it, we also showed the measured Ct values in residual plot relative to the fitted straight line (Supplementary Fig. 3b), and the plots showed how the spread of replicates increases with decreasing number of targets as that of the previously published tests [21]. Although other factors contribute to variation across replicates [28], sampling noise, which can be modeled by the Poisson distribution, is expected to dominate at very low copy numbers. In the future, it is recommended to increase the number of duplicates at very low copy numbers to further improve the data quality.

Taken together, considering the fact that rRT-PCR is the most popular MDx system and SENA is simple to operate, SENA has the potential to be widely used in various scenarios to solve the uncertainty,
problems of RT-PCR as well as other nucleic acid amplification-based MDx. Of course, to minimize the possibility of aerosol contamination during opening of the RT-PCR tubes and pipetting, physical separation of the SENA detection center from the clinical PCR laboratory is an absolute requisition. Abided by this rule, SENA has been demonstrated convenient and effective in several hospitals and centers for disease control and prevention as part of their laboratory routine in combination with rRT-PCR for more sensitive and accurate detection of SARS-CoV-2 infection. Therefore, SENA is a useful technique that meets the urgent needs of combating COVID-19 pandemic.

Data sharing

All data is available in the main text or the Supplementary materials.

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Declarations of Interests

G.Z. and J.W. are cofounders of Tolo Biotechnology Co., Ltd., W.H., G.Z. and J.W. have filed patent applications relating to the work in this manuscript. All other authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2020.103036.

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