SARS-CoV-2 detection with CRISPR diagnostics

Author list: Lu Guo¹,²,³,* , Xuehan Sun¹,²,³,* , Xinge Wang¹,²,³,* , Chen Liang¹,²,³,* , Haiping Jiang¹,²,³,* , Qingqin Gao¹,²,³,* , Moyu Dai¹,²,³,* , Bin Qu¹,²,³ , Sen Fang¹,²,³ , Yihuan Mao¹,²,³ , Yangcan Chen¹,²,³ , Guihai Feng¹,² , Qi Gu¹,² , Liu Wang¹,² , Ruiqi Rachel Wang¹,²,# , Qi Zhou¹,²,³,# , Wei Li¹,²,³,#

Affiliations:

1State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China.
2Institute for Stem Cell and Regeneration, Chinese Academy of Sciences, Beijing 100101, China.
3University of Chinese Academy of Sciences, Beijing 100049, China.

*These authors contributed equally to this work.

#Correspondence: Ruiqi Rachel Wang (wangrr@ioz.ac.cn); Qi Zhou (zhouqi@ioz.ac.cn); or Wei Li (liwei@ioz.ac.cn)
Abstract

The novel coronavirus (CoV) disease termed COVID-19 (Coronavirus Disease-19) caused by SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus-2) is causing a massive pandemic worldwide, threatening public health systems across the globe. During this ongoing COVID-19 outbreak, nucleic acid detection has played an important role in early diagnosis. Here we report a SARS-CoV-2 detection protocol using a CRISPR-based CRISPR diagnostic platform - CDetection (Cas12b-mediated DNA detection). By combining sample treatment protocols and nucleic acid amplification methods with CDetection, we have established an integrated viral nucleic acid detection platform - CASdetec (CRISPR-assisted detection). The detection limit of CASdetec for SARS-CoV-2 pseudovirus is $1 \times 10^4$ copies/mL, with no cross reactivity observed. Our assay design and optimization process can provide guidance for future CRISPR-based nucleic acid detection assay development and optimization.

Results

The novel coronavirus (CoV) disease termed COVID-19 (Coronavirus Disease-19) caused by SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus-2) [1] is causing a massive pandemic worldwide, threatening public health systems across the globe. During this ongoing COVID-19 outbreak, nucleic acid detection has played an important role in early diagnosis [2]. To date, 4 protocols based on CRISPR for detecting SARS-CoV-2 have been published [3-6]. Using lateral flow protocols, RNA samples harboring more than $1 \times 10^4$ - $1 \times 10^5$ copies/mL (SHERLOCK) or $1 \times 10^4$ copies/mL (DETECTR) can be detected within 1 hour. In addition to these reported efforts, we have also established a SARS-CoV-2 detection protocol based on our previously reported platform – CDetection (Cas12b-mediated DNA detection) [7]. By combining sample treatment protocols and nucleic acid amplification methods with CDetection, we have established an integrated viral nucleic acid detection platform – CASdetec (CRISPR-assisted detection). The detection limit of CASdetec for SARS-CoV-2 pseudovirus is $1 \times 10^4$ copies/mL, with no cross reactivity observed. Here we present our assay design and optimization process, which could provide guidance for future CRISPR-based nucleic acid detection assay development and optimization.

To optimize the output of fluorescence signal, we designed and synthesized poly-T
fluorescence-quenchers of varying nucleotide lengths, namely 4 nt, 5 nt, 7 nt, 12 nt, 17 nt, 22 nt and 27 nt. Of all the lengths tried, the 7 nt poly-T reporter provided the highest signals in the shortest amount of time (Supplementary information, Fig. S1a, b). Based on this observation, we applied the 7 nt poly-T reporter in later experiments.

According to the published SARS-CoV-2 whole genome sequence [8], we designed 7 sgRNAs around the RdRp locus, as recommended by the World Health Organization (WHO) [2] (Supplementary information, Fig. S2a). Due to its high similarity to SARS-CoV, we ran initial experiments on both SARS-CoV-2 and SARS-CoV plasmids. According to fluorescence kinetics studies, sgRNA-3 stood out in not only being able to distinguish between the 2 similar coronaviruses but also being able to produce the most distinct fluorescence signal (Fig. 1a and Supplementary information, Fig. S2b-g).

As previously demonstrated, CRISPR is unable to detect any target DNA when there is less than 1-10 nM of amplification product within the reaction mix [7]. Hence, increasing the molecular collisions between CRISPR and target would be essential to improve sensitivity. We found out that increasing the sgRNA concentration by 3 folds not only enhances the fluorescence signal and the signal-to-background ratio, but also increases the rate of reaction (Fig. 1b and Supplementary information, Fig. S3a-b).

Given that the average viral load in the plasma of SARS patients ranged from less than 1 to about 1000 copies per microliter [9], or $1 \times 10^3 - 1 \times 10^6$ copies/mL, nucleic acid amplification techniques are needed to produce sufficient DNA for CRISPR-based DNA detection methods. Recombinase-aided amplification (RAA) can amplify substrates $10^{10}$ times at most (from aM to 10 nM) within 10-30 minutes at constant temperature between 37°C to 42°C, complementing the needs of CRISPR-based detection. Thus, we designed and screened RAA primers that matched our previously optimized sgRNA-3 (Supplementary information, Fig. S4a). Based on our screens, we found that by using the best primer pairs together with sgRNA-3, we can detect SARS-CoV-2 RNA in samples containing $5 \times 10^3$ copies/mL (Fig. 1c and Supplementary information, Fig. S4b-c).

In addition, we aligned the selected primers and sgRNAs to the existing typical coronavirus sequences to evaluate their specificity. We analyzed all SARS-CoV-2 sequences that have been uploaded to GISAID up till March 26th 2020. Out of 1792 sequences on
GISAID, 1673 of them contained sequences matching our chosen primers and sgRNA. Only 3 of them have 1 mismatch to the forward primer and only 2 of them have 1 mismatch to the reverse primer (Supplementary information, Fig. S5), suggesting that our selected sgRNA and primers can be used for nearly all of the reported SARS-CoV-2 genomes. Meanwhile, we aligned 12 typical human coronaviruses, and found that none of the whole set of primers and sgRNA showed high similarity (Supplementary information, Fig. S6a-c).

The experiments above were conducted by executing RT-RAA nucleic acid amplification and CDetection separately. However, it would be best to conduct both reactions within a single tube for convenience and, more importantly, to prevent aerosol contamination which happens when the reaction mixture has to be exposed to the environment midway through the protocol. Hence, we tried to execute both the RT-RAA and CDetection concurrently within a single tube. However, the combination resulted in a drastic decrease in sensitivity (Fig. 1c and Supplementary information, Fig. S7). Therefore, in order to keep both the RT-RAA and CRISPR reactions within a single tube, we executed the RT-RAA reaction within the tube while keeping the CDetection reagents within the lid of the tube for 30 minutes, following which, the CDetection reagents were spun down into the tube for nucleic acid detection, and the resultant reaction mixture was imaged for fluorescence.

To validate the specificity of our method for SARS-CoV-2 nucleic acid detection, we tested our protocols against 6 coronaviruses known to cause respiratory diseases (SARS-CoV, MERS-CoV, CoV-HKU1, CoV-229E, CoV-OC43 and CoV-NL63). Consistent with alignment analysis (Supplementary information, Fig. S6a-c), no cross-reactivity with other endemic human coronavirus were detected (Fig. 1d). Our results suggested our set of sgRNA and primers showed high sensitivity and specificity.

However, viral genomes are packaged inside capsid protein and need to be released. Thus, to investigate the virus handling processes, we produced pseudoviruses by packaging the target sequences of SARS-CoV-2, SARS-CoV, MERS-CoV into actual lentivirus particles (Supplementary information, Fig. S8). These pseudoviruses were diluted serially and treated with either virus genome extraction kits (spin column) or lysis buffer, respectively. Our results demonstrated that the spin column treatment gave a lower detection limit of $1 \times 10^4$ copies/mL (Fig. 1e). On the other hand, the lysis buffer offered higher usability, but raised the
detection limit to $5 \times 10^4$ copies/mL (Fig. 1f). Due to the difference in detection limit between spin column and lysis buffer, we suggest using the spin columns in hospitals, and using the lysis buffer for point-of-care testing (POCT).

To make CASdetec more amenable for POCT, we have also constructed a portable dark box containing a blue LED and demonstrated that the positive fluorescence signal generated from the protocol can be visualized upon illumination by a blue LED (Fig. 1g). In conclusion, we have established a CRISPR-assisted detection (CASdetec) platform which consists of procedures including virus handling, nucleic acid amplification and CRISPR-based detection (Fig. 1h). CASdetec can detect pseudovirus samples with more than $1 \times 10^4$ copies/mL, with no cross-reactivity with other endemic human coronaviruses. In addition, we optimized the workflow to run both reactions within one single tube without lid opening. This will thus prevent aerosol contamination and reduce the false positive rate.

Acknowledgments

We thank Prof. Ng Shyh-Chang from Institute of Zoology, CAS for his critical support with this study. We thank Hanxing Zhang from Institute of Microbiology, CAS for her kind help on equipment. This work was supported by the National Key Research and Development Program (2020YFA070009 to R.R.W), the Key Research Projects of the Frontier Science of the Chinese Academy of Sciences (QYZDY-SSW-002 to Q.Z. and QYZDB-SSW-002 to W.L.), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA16030400 to W.L.).

Author contributions

L.G., R.R.W., Q.Z., and W.L. conceived and designed the experiments. L.G., X.S., X.W., C.L., H.J., Q.G., M.D., B.Q., S.F., Y.M. and Y.C. participated in multiple experiments; L.G., X.W., X.S., C.L., and G.F. analyzed the data. L.G. wrote the manuscript. W.L., R.R.W., Q.Z., and L.W. provided the final approval of the manuscript.

Figure legends

Figure 1 CASdetec used for SARS-CoV-2 detection. (A) Fluorescence kinetics of
sgRNA-3 for RdRp detection. *E. coli* cells bearing Blunt-SARS-CoV-RdRp or Blunt-SARS-CoV-2-RdRp were pre-incubated at 95°C for 10 min and used as templates for RAA and CDetection. PAM sequences are colored in green, protospacers are colored in blue, base pair mismatches are colored in red. Error bars indicate standard errors of the mean (s.e.m.), *n* = 3. RFU, relative fluorescence units. **(B)** Fluorescence kinetics of RdRp detection using 108 nM sgRNA-3. Plasmid bearing SARS-CoV-2-RdRp was serially diluted as shown in the legend. *n* = 2. ΔRn, ΔFluorescence, which refers to the Rn value of an experimental reaction minus the Rn value of the baseline signal generated by ABI 7500. **(C)** Fluorescence kinetics of F1- and R1-based RdRp detection. SARS-CoV-2-RdRp RNA was serially diluted as shown in the legend. Error bars indicate (s.e.m.), *n* = 3. **(D)** Evaluation of cross reactivity. Plasmids containing target RdRp region from 6 human epidemic coronaviruses are serially diluted as shown in the legend. *n* = 2. **(E)** Detection of SARS-CoV-2 pseudovirus. Virus genome is extracted using the virus RNA extraction kit (spin column). SARS-CoV were diluted to 5 × 10⁵ copies/mL. *n* = 2. **(F)** Detection of SARS-CoV-2 pseudovirus. Virus was treated by direct lysis. SARS-CoV was diluted to 5 × 10⁵ copies/mL. *n* = 2. **(G)** CASdetec results can be directly observed under blue LED. 3 replicates of product from Fig. 1b are imaged upon blue LED illumination. **(H)** Schematics showing the workflow of CASdetec. Virus genome is extracted by kit or direct lysis. Target sequences are pre-amplified by isothermal amplification, followed is CDetection. Fluorescence signals are obtained either from fluorescence reader or direct observation under blue light.
References

1. Coronaviridae Study Group of the International Committee on Taxonomy of Viruses. The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat Microbiol* 2020; 5: 536-544.

2. Corman VM, Landt O, Kaiser M et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill* 2020; 25.

3. Zhang F, Abudayyeh OO, Gootenberg JS. A protocol for detection of COVID-19 using CRISPR diagnostics. 2020
   https://www.broadinstitute.org/files/publications/special/COVID-19%20detection%20(updated).pdf

4. Metsky HC, Freije CA, Kosoko-Thoroddsen et al. CRISPR-based COVID-19 surveillance using a genomically-comprehensive machine learning approach. *bioRxiv* 2020: 2020.2002.2026.967026.

5. Lucia C, Federico PB, Alejandra GC. An ultrasensitive, rapid, and portable coronavirus SARS-CoV-2 sequence detection method based on CRISPR-Cas12. *bioRxiv* 2020: 2020.2002.2029.971127.

6. Broughton JP, Deng X, Yu G et al. Rapid Detection of 2019 Novel Coronavirus SARS-CoV-2 Using a CRISPR-based DETECTR Lateral Flow Assay. *medRxiv* 2020: 2020.2003.2006.20032334.

7. Teng F, Guo L, Cui T et al. CDetection: CRISPR-Cas12b-based DNA detection with sub-attomolar sensitivity and single-base specificity. *Genome Biol* 2019; 20: 132.

8. Zhang Y. Novel 2019 coronavirus genome. 2020
   http://virological.org/t/novel-2019-coronavirus-genome/319

9. Grant PR, Garson JA, Tedder RS et al. Detection of SARS coronavirus in plasma by real-time RT-PCR. *N Engl J Med* 2003; 349: 2468-2469.
SARS-CoV-2
5' - ……TTCACTCAATACTTGAGCACACT……-3'

SARS-CoV
5' - ……CTCACTTAATACTTGCGCACACT……-3'

sgRNA-3
5' - ……ACTCAATACTTGAGCACACT-3'

RFU
0
5000
10000
15000
20000

Time (min)
0
20
40
60
80
100
120

E. coli
SARS-CoV-2
SARS-CoV
Plasmid
copies/ml
1 × 10^5
5 × 10^4
1 × 10^4
5 × 10^3
1 × 10^3
0

RNA (copies/ml)
1 × 10^6
5 × 10^5
1 × 10^5
5 × 10^4
1 × 10^4
5 × 10^3
1 × 10^3
0