

Genetic variation of coronavirus SARS-CoV-2 in target regions of CRISPR-based diagnostic assays

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; initially named as 2019-nCoV) is the cause of the novel coronavirus disease 2019 (COVID-19) pandemic. Its diagnosis relies on the molecular detection of the viral RNA by polymerase chain reaction (PCR) while newer rapid CRISPR-based diagnostic tools are being developed for point-of-care and in-home diagnosis. As molecular diagnostic assays rely on the detection of unique sequences of viral nucleic acid, these are prone to mismatches due to mutations in the viral genome. This stage 1 protocol proposes the verification of the sequence variability within the target regions of recently developed CRISPR-based diagnostic assays in the SARS-CoV-2 genome. This would be accomplished using more than 60 000 publicly available sequences of viral isolates from around the world. The absence of any mutations in target regions of the assay used would provide a higher degree of confidence in the alternative tests being developed while the presence of mutations could help guide the assay development efforts. We believe that this study would provide potentially important information for clinicians, researchers, and policy-makers.

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Abstract:

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; initially named as 2019-nCoV) is the cause of the novel coronavirus disease 2019 (COVID-19) pandemic. Its diagnosis relies on the molecular detection of the viral RNA by polymerase chain reaction (PCR) while newer rapid CRISPR-based diagnostic tools are being developed for point-of-care and in-home diagnosis. As molecular diagnostic assays rely on the detection of unique sequences of viral nucleic acid, these are prone to mismatches due to mutations in the viral genome. This stage 1 protocol proposes the verification of the sequence variability within the target regions of recently developed CRISPR-based diagnostic assays in the SARS-CoV-2 genome. This would be accomplished using more than 60 000 publicly available sequences of viral isolates from around the world. The absence of any mutations in target regions of the assay used would provide a higher degree of confidence in the alternative tests being developed while the presence of mutations could help guide the assay development efforts. We believe that this study would provide potentially important information for clinicians, researchers, and policy-makers.

Keywords: SARS-CoV-2, coronavirus, sequence variation, mutations, diagnosis, CRISPR, COVID-19

Short Title: Mutations in CRISPR-based COVID-19 tests

Introduction:

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; initially named as 2019-nCoV) was firstly isolated from a cluster of pneumonia patients in Wuhan, China and is the cause of novel coronavirus disease termed COVID-19 (Wu et al. 2020; Zhou et al. 2020; Zhu et al. 2020). The rapid spread of the virus has resulted in a global pandemic reaching more than 200 countries and territories (Worldometers.info 2020). SARS-CoV-2 has been classified as a member of family *Coronaviridae* in the genus *Betacoronavirus* along with SARS-CoV and Middle East respiratory syndrome (MERS)-CoV (Gorbalenya et al. 2020). The sequencing of the virus from the patients early in the outbreak has shown that its single-stranded RNA genome is ~30 kb in size (Chan et al. 2020; Lu et al. 2020; Wu et al. 2020). SARS-CoV-2 genome has been predicted to encode at least 10 open reading frames (ORFs) for structural and accessory proteins, based on similarity with SARS-CoV. As per current annotation (NC_045512.2), these viral ORFs encode replicase ORF1ab, spike (S), envelope (E), membrane (M) and nucleocapsid (N), and at least 6 accessory proteins (3a, 6, 7a, 7b, 8, and 10) (NCBI 2020).

The pandemic has serious public health and economic implications. The day-to-day life of billions of people has been affected due to different forms of social distancing measures in place in different parts of the world to mitigate the spread of the virus. Thus, the widespread availability of rapid and reliable diagnostic testing is an important tool for policymakers to make public health decisions. The current diagnosis of COVID-19 relies on the molecular detection of the viral RNA from the patient samples using nucleic acid amplification tests (NAAT) like polymerase chain reaction (PCR) (WHO 2020). However, PCR requires specialized equipment and trained staff to perform the test and interpret the results and thus is a challenge for remote low-resource settings (Giri & Rana 2020). One of the alternatives being explored is the CRISPR-based nucleic-acid detection methods that may be particularly useful for point-of-care and in-house diagnosis. CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR-associated), a component of the bacterial immune system to infectious nucleic acid, has been widely used as a gene-editing tool. This technology exploits the ability of Cas proteins to accurately target any region in DNA in association with CRISPR RNA (crRNA) that matches the target DNA with or without the requirement of a protospacer adjacent motif (PAM) (Moon et al. 2019). Initially explored for Cas9 protein, the application of CRISPR in nucleic acid detection

emerged as a viable tool with the discovery of promiscuous collateral cleavage activity of Cas12a (also known as C2c2) and Cas13a (also known as Cpf1) after target recognition (Chen et al. 2018; Gootenberg et al. 2017). Subsequently, Cas14 was also shown to have this property (Harrington et al. 2018). Several CRISPR-based methods have been developed for the detection of RNA and DNA viruses (reviewed in (Jia et al. 2020; Strich et al. 2019)). With the emergence of the novel coronavirus, scientists are rapidly employing these tools for the detection of SARS-CoV-2 from patient samples as an alternative to PCR. The Cas12a based DETECTR assay has been employed for diagnosis of COVID-19 from patient samples targeting viral genes N and E (Broughton et al. 2020). Similarly, Cas12b based CASdetec has also been developed for the detection of SARS-CoV-2 (Guo et al. 2020). Cas13a based SHERLOCK has also been approved as the first CRISPR-based assay by the US Food and Drug Administration (FDA) under Emergency Use Authorization (Sherlock 2020). Several other CRISPR-based methods are also being developed (Esbin et al. 2020).

The molecular diagnosis of SARS-CoV-2 may be jeopardized by potential preanalytical and analytical vulnerabilities leading to false-positive or false-negative results (Lippi et al. 2020). As molecular diagnostic assays rely on the detection of unique sequences of viral nucleic acid, these are prone to mismatches due to genetic variability in the viral genome. It is known that mutations at primer/probe binding regions of the viral genome can result in potential mismatches and false-negative PCR diagnosis (Lefever et al. 2013; Stadhouders et al. 2010; Whiley & Sloots 2005). We have demonstrated the genetic variability in the primer/probe binding regions of the SARS-CoV-2 genome using more than 17 000 sequences highlighting the importance of periodic sequence verification for optimal virus detection (Khan & Cheung 2020). Although analyzed much fewer sequences, other concurrent studies reached the same conclusion (Farkas et al. 2020; Osorio & Correia-Neves 2020). One of the focus of CRISPR-diagnostics remained to improve assay specificity as tolerance of mismatches by Cas9 can result in a false-positive diagnosis. This risk has been minimized with the newer Cas proteins, Cas12 and Cas13, that have a lower tolerance for mismatches compared to Cas9 especially in the “seed” region (Safari et al. 2019). *Francisella novicida* Cas9 (FnCas9) has been reported to have higher specificity and lower tolerance for mismatches compared to *Streptococcus pyogenes* Cas9 (SpCas9) especially at the PAM-distal end (Acharya et al. 2019). However, this raises the possibility that these tests may miss certain viral variants due to genetic variability in the regions targeted by these assays.

Mismatch intolerant seed region of ~6 nucleotides is located in the PAM-proximal region for Cas12 (Chen et al. 2018; Kim et al. 2016) while the seed region is located in the central region of crRNA for Cas13a (Abudayyeh et al. 2017; Cox et al. 2017).

The objective of this study is the verification of the sequence variability within the target regions of recently developed CRISPR-based COVID-19 diagnostic assays using publicly available SARS-CoV-2 sequences from around the world. The absence of any mutations in target regions of the assay used would provide a higher degree of confidence in the alternative tests being developed while the presence of mutations could help guide the assay development efforts. We believe that this study would provide potentially important information for clinicians, researchers and policy-makers.

Methods:

CRISPR-based diagnostic assays and SARS-CoV-2 sequences

At least 10 crRNA of recently published* CRISPR-based methods would be selected based on the literature review. The sequence variability would be determined in the publicly available viral sequences within the target regions of CRISPR-based diagnostic assays using the protocol described previously (Khan 2020; Khan & Cheung 2020). The summary of the sequence tracing pipeline is shown in Figure 1. The design planner is included in Table 1.

Complete genome sequences of the virus would be obtained from the Global Initiative on Sharing All Influenza Data (GISAID) EpiCoV database (Shu & McCauley 2017) that are available upon free registration (<https://www.gisaid.org/>). A total of 60 000** near full-length sequences would be downloaded by applying the full length filter (>29,000bp). The RNA genome of SARS-CoV-2 is shown in DNA format as per scientific convention. The viral sequences are deposited in GISAID EpiCoV database by the laboratories around the world and a list of accession numbers would be included as supplemental data.

* Assay published in the coming weeks would also be included.

** The number of sequences in EpiCoV database is growing on a daily basis and the exact number of included sequences would be updated in the 2nd stage submission.

Multiple sequence alignment and alignment processing

Multiple sequence alignment (MSA) would be performed using MAFFT (Multiple Alignment with Fast Fourier Transform) program version 7 dedicated to closely-related viral genomes available online (<https://mafft.cbrc.jp/alignment/software/closelyrelatedviralgenomes.html>) (Kato et al. 2002; Kato et al. 2019). The complete genome of Wuhan-Hu-1, which is 29,903 bp long, would be downloaded from NCBI nucleotide and would be included as a reference (NCBI Reference Sequence: NC_045512.2). The aligned sequences would be downloaded in PIR format and the alignment would be visually inspected using the AliView program 1.26 (Larsson 2014). MAFFT online service dedicated to MSA of closely-related viral genomes supports up to 20 000 sequences of ~30 kb in length. Thus the tracing pipeline would be applied to sequences in batches of less than 20 000 sequences and results would be subsequently combined. To evaluate the sequence variability in target regions referred here as the region of interest (ROI), the sequence targeted by each assay will be saved as a separate file in FASTA format.

Sequence variation in target regions of CRISPR-based assays

The MSA sequence for each ROI would be stratified using the SequenceTracer module (<http://entropy.szu.cz:8080/EntropyCalcWeb/sequences>) of the Alignment Explorer (Nagy et al. 2019). This tool segregated sequences into discrete groups of identical sequence variants along with their frequency. SequenceTracer automatically segregates the sequences with ambiguous sequences, stretches of NNNs, and missing sequences in ROI and these sequences will be excluded from the analysis. To remove extremely low prevalent variants and sequencing errors in the data, only the sequence variants occurring more than once would be further considered (Bioinformatics-Institute 2020). The viral isolates would be reported as the frequency of hits with 100% match and hits with mismatches along with a summary of mutated nucleotides for each crRNA. In addition, the frequency of sequence variants with mismatches in the seed and PAM region of each crRNA would be determined. The base composition of each nucleotide position of highly variable regions, if any, would further be analyzed using the positional

nucleotide numerical summary (PNNS) calculator
(<http://entropy.szu.cz:8080/EntropyCalcWeb/pnns>) of the Alignment Explorer (Nagy et al.
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Table 1: Study design planner.

Question	Hypothesis	Sampling plan (e.g. power analysis)	Analysis Plan	Interpretation given different outcomes
Are there any mutations in the SARS-CoV-2 genome within the target regions of CRISPR-based COVID-19 diagnostic assays?	Given the potential of virus to mutate during natural evolution, genetic variability in the target regions of CRISPR-based COVID-19 diagnostic assays can result in crRNA-template mismatches.	<p>>60 000* viral isolates would be download from GISAID EpiCov database.</p> <p>*The number of sequences is growing on a daily basis and the exact number of included sequences would be updated in the 2nd stage</p> <p><u>Inclusion criteria</u> Only full length (>29,000 bp)</p> <p><u>Exclusion Criteria</u> The sequences with stretches of NNNs, ambiguous sequences, and missing sequences in the region of interest (ROI) will be considered low quality and would be excluded.</p>	<p>- Sequences would be aligned using MAFFT</p> <p>- The alignment would be inspected visually using AliView</p> <p>- Sequence variability would be traced using SequenceTracer.</p> <p>- The highly variable region, if any, would be further analyzed for nucleotide composition at each position using positional nucleotide numerical summary (PNNS)</p> <p>- The complete genome of Wuhan-Hu-1 would act as a positive control (NCBI Reference Sequence: NC_045512.2).</p>	<p>- In the event of a negative result, it would be concluded that there is no evidence of a difference between crRNA and viral isolates.</p> <p>- This would serve as a reference for clinicians and researchers using CRISPR-based assays for the detection of SARS-CoV-2.</p>

Figure 1: Sequence tracing pipeline to be used in the study. Adapted from (Khan & Cheung 2020) shared under a CC BY 4.0 license (<https://creativecommons.org/licenses/by/4.0/>).

Figure 1

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