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Targeted Amplification and Genetic Sequencing of the Severe Acute Respiratory Syndrome Coronavirus 2 Surface Glycoprotein

Matthew Keller, Lisa Keong, Benjamin Rambo-Martin, Norman Hassell, Kristine Lacek, Malania Wilson, Marie Kirby, Jimma Liddell, D Owuor, Mili Sheth, Joseph Madden, Justin Lee, Rebecca Garten Kondor, David Wentworth, and John Barnes

Corresponding Author(s): Matthew Keller, Centers for Disease Control and Prevention

Review Timeline:

Submission Date:	September 6, 2023
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Reviewer(s): Disclosure of reviewer identity is with reference to reviewer comments included in decision letter(s). The following individuals involved in review of your submission have agreed to reveal their identity: Michael Owusu (Reviewer #2)

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

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October 9, 2023

Dr. Matthew W Keller
Centers for Disease Control and Prevention
Atlanta, GA

Re: Spectrum02982-23 (Targeted Amplification and Genetic Sequencing of the Severe Acute Respiratory Syndrome Coronavirus 2 Surface Glycoprotein)

Dear Dr. Matthew W Keller:

This is a strong project nonetheless but perhaps, could be presented in a better way. The group needs to transfer some supplemental data into figures for the main text and in some cases, elaborate and describe the data a bit more and not assume the audience can understand the data.

Thank you for submitting your manuscript to Microbiology Spectrum. When submitting the revised version of your paper, please provide (1) point-by-point responses to the issues raised by the reviewers as file type "Response to Reviewers," not in your cover letter, and (2) a PDF file that indicates the changes from the original submission (by highlighting or underlining the changes) as file type "Marked Up Manuscript - For Review Only". Please use this link to submit your revised manuscript - we strongly recommend that you submit your paper within the next 60 days or reach out to me. Detailed instructions on submitting your revised paper are below.

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Sincerely,

Day-Yu Chao

Editor, Microbiology Spectrum

Journals Department
American Society for Microbiology
1752 N St., NW
Washington, DC 20036
E-mail: spectrum@asmusa.org

Reviewer comments:

Reviewer #1 (Comments for the Author):

Thank you for the opportunity to review this manuscript. In this study, Keller et al. describe the challenges associated with sequencing the SARS-CoV-2 S-gene in the context of mutations across the COVID-19 pandemic, and describe the development and validation of a targeted method to amplify and sequence the S-gene. Furthermore, the authors explain the potential public health impact of this method due to the fact that the total number of primers used is four, and the number of primers within the coding region of the S-gene is two. This is a well-written manuscript that contributes to the growing body of COVID-19 evidence, and has an important focus on accessibility, surveillance, and global public health.

Major Concerns: None

Minor Concerns:
1.Line 175-176: If possible, it would be great if a citation could be added to the end of this sentence to elucidate/support the minimum amount of mutations needed to be considered acceptable for surveillance (or something that references public health sequencing recommendations).

2.Next generation sequencing is abbreviated throughout the manuscript. Consider doing the same with whole-genome sequencing, if appropriate.

Reviewer #2 (Comments for the Author):

Authors have described a robust method to target the S-gene for
amplification and sequencing of SARS-CoV-2. The methods appear useful in making decisions on virus mutations and evolutions of essential regions such as the spike protein.The manuscript generally appears sound with good technical and scientific details. Authors have indicated progress toward making these methods available to LMICs.
Authors have managed to show data on LOD and sequence coverage for FLG and MIN but not for the illumina based sequences. It would be good for authors to describe three-way analysis and graphical representation of the LODs, depth of coverage and sequence quality. Authors should refer to FIG S18 - FIG S23 to check how a third arm of sequence analysis from Illumina could be included.

Reviewer #3 (Public repository details (Required)):

The group performed sequencing of SARS-CoV-2 isolates which should include a large dataset that needs to be deposited.

Reviewer #3 (Comments for the Author):

Keller et al. describe in their manuscript the development of a new SARS-CoV-2 sequencing protocol by amplifying and sequencing the S-gene. They showed that their primers allow for detection of all the variant waves of COVID-19 even up to the Omicron variant as of July 2023 and validated their design by testing 321 matched samples. Their assay can be used on the Oxford Nanopore platform which can even be applied in low research settings.

Major Comments:
1. There is quite a bit of data generated and work put into this project. However, all the data in the 'Results' section are in the Supplemental Material. This makes it very difficult for the readers to grasp the material and truly appreciate the work and findings. Please consider moving some of the supplemental data into the main text. I think some of the key figures from the supplemental data include the conservation of your primers (lines 125-133), the LoD, Method Validation (line 149), and Phylogenetics (line 212). Rather than putting all the data, please find representative data per each section of the 'Results' section to make different figures. Then, any extra data can be referred to in Supplemental Material.

2. Certain areas of the 'Results' can be further elaborated and described. For example, lines 138-140 where it simply states "We performed 14 Nanopore sequencing runs to validate and characterize our method. A summary of these runs is available in the supplemental materials" is not sufficient for the 'Results' section paragraph and should really be elaborated given that these runs are important for the paper.

3. The abstract states, "While it is adaptable to other sequencing platforms, the Nanopore platform validated here...". I would suggest making a separate figure and section in the 'Results' that compares performance of different platforms. If not, then this statement in the 'Abstract' is an overstatement and this manuscript should mainly be focused on Oxford Nanopore technologies, which has merit in itself as well. Please revise accordingly.

4. Much of the 'Discussion' was written as a description for 'ongoing projects' and 'future steps' with this project, which is acceptable. However, some of the key components for a typical 'discussion' section appears to be missing. For example, how does your protocol compare to other assays ran on Oxford Nanopore? What are the limitations of this study?

Minor comments:
1. Some of the supplemental data has the Qiagen header in the data and it appears like these are raw printouts of the data. Is this allowed for publication since Qiagen's logo and trademark is being used? Please present the data in another way and make your own figures with the raw data.

Staff Comments:

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• Each figure must be uploaded as a separate file, and any multipanel figures must be assembled into one file.
• Manuscript: A .DOC version of the revised manuscript
• Figures: Editable, high-resolution, individual figure files are required at revision, TIFF or EPS files are preferred

For complete guidelines on revision requirements, please see the journal Submission and Review Process requirements at https://journals.asm.org/journal/Spectrum/submission-review-process. **Submissions of a paper that does not conform to Microbiology Spectrum guidelines will delay acceptance of your manuscript.** "

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Thank you for submitting your paper to Microbiology Spectrum.

Summary of Key Findings (200-250 words)
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Major Concerns (at most 5-6): None

Minor Concerns (at most 5-20 in bullet points):

1. Line 175-176: If possible, it would be great if a citation could be added to the end of this sentence to elucidate/support the minimum amount of mutations needed to be considered acceptable for surveillance (or something that references public health sequencing recommendations).
2. Next generation sequencing is abbreviated throughout the manuscript. Consider doing the same with whole-genome sequencing, if appropriate.

Editor

This is a strong project nonetheless but perhaps, could be presented in a better way. The group needs to transfer some supplemental data into figures for the main text and in some cases, elaborate and describe the data a bit more and not assume the audience can understand the data.

We thank the editor for the comments and for refereeing this review. We too believe this is a strong project. To clarify the work done, we have incorporated much of the suggested feedback. Primarily, we have moved some figured from the supplemental into the main text and have expanded the results sections discussing those key finding. We have also expanded the discussion to include limitations. We disagree with reviewer #2 regarding the inclusion of LOD data for a third sequencing method. We have elaborated in the response to that comment, but to briefly summarize, we feel that experiment would not reveal any practical information as the LOD is primarily a property of the RT-PCR and the influence of a different sequencer has less influence than how many samples are indexed for a single run.

Reviewer #1 (Comments for the Author):

Thank you for the opportunity to review this manuscript. In this study, Keller et al. describe the challenges associated with sequencing the SARS-CoV-2 S-gene in the context of mutations across the COVID-19 pandemic, and describe the development and validation of a targeted method to amplify and sequence the S-gene. Furthermore, the authors explain the potential public health impact of this method due to the fact that the total number of primers used is four, and the number of primers within the coding region of the S-gene is two. This is a well-written manuscript that contributes to the growing body of COVID-19 evidence, and has an important focus on accessibility, surveillance, and global public health.

Major Concerns: None

Minor Concerns:

1.Line 175-176: If possible, it would be great if a citation could be added to the end of this sentence to elucidate/support the minimum amount of mutations needed to be considered acceptable for surveillance (or something that references public health sequencing recommendations).

As suggested, we've added a reference to Genomic sequencing of SARS-CoV-2: A guide to implementation for maximum impact on public health.

2.Next generation sequencing is abbreviated throughout the manuscript. Consider doing the same with whole-genome sequencing, if appropriate.

We thank the reviewer for the suggestion. Whole-genome sequencing has been abbreviated to WGS throughout the manuscript

Reviewer #2 (Comments for the Author):

Authors have described a robust method to target the S-gene for amplification and sequencing of SARS-CoV-2. The methods appear useful in making decisions on virus mutations and evolutions of essential regions such as the spike protein.The manuscript generally appears sound with good technical and scientific details. Authors have indicated progress toward making these methods available to LMICs.

Authors have managed to show data on LOD and sequence coverage for FLG and MIN but not for the illumina based sequences. It would be good for authors to describe three-way analysis and graphical representation of the LODs, depth of coverage and sequence quality. Authors should refer to FIG S18 - FIG S23 to check how a third arm of sequence analysis from Illumina could be included.

We thank the reviewer for this suggestion. LOD is an important characteristic to understand the practicality of a test. Still, is it difficult to distinguish the relative sensitivity of this method on differing sequencing platforms because the primary driver of sensitivity is the RT-PCR. Moreover, sequencer sensitivity can vary based on the number of samples indexed on any given run.

Illumina and flongle sequencing played discrete but secondary roles in this work. We characterized the LOD and yield for FLG because of our intention to supply partner labs with those flow cell types. The role of illumina sequencing in this manuscript is to evaluate the accuracy of the nanopore sequencing. Illumina is widely regarded as the gold standard of NGS for accuracy. Therefore, it was included here to support our claims that nanopore sequencing can accurately identify mutations in the S-gene.

A meaningful characterization of sensitivity goes beyond testing serial dilutions. For that reason, we have included, in our original submission, a comparison of sequencing results versus Ct value for 277 clinical specimens. To highlight this experiment and further clarify the sensitivity characterization, we have moved those results into the main text and expanded the results section describing those results.

Reviewer #3 (Public repository details (Required)):

The group performed sequencing of SARS-CoV-2 isolates which should include a large dataset that needs to be deposited.

We thank the reviewer for this important comment. We agree that sequencing data should be deposited online. Raw sequencing data is deposited online at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA999712. A supplemental table was added that contains the consolidate metadata, biosample, and SRA identifiers associated with that raw sequencing data.

Reviewer #3 (Comments for the Author):

Keller et al. describe in their manuscript the development of a new SARS-CoV-2 sequencing protocol by amplifying and sequencing the S-gene. They showed that their primers allow for detection of all the variant waves of COVID-19 even up to the Omicron variant as of July 2023 and validated their design by testing 321 matched samples. Their assay can be used on the Oxford Nanopore platform which can even be applied in low research settings.

Major Comments:

1. There is quite a bit of data generated and work put into this project. However, all the data in the 'Results' section are in the Supplemental Material. This makes it very difficult for the readers to grasp the material and truly appreciate the work and findings. Please consider moving some of the supplemental data into the main text. I think some of the key figures from the supplemental data include the conservation of your primers (lines 125-133), the LoD, Method Validation (line 149), and Phylogenetics (line 212). Rather than putting all the data, please find representative data per each section of the 'Results' section to make different figures. Then, any extra data can be referred to in Supplemental Material.

We thank the reviewer for this important comment. We agree that the findings can be better explained by moving key figures into the main text and expanding the results sections describing those figures. To

address this we have: added a multipaned figure demonstrating the conservation of the primers (figure 3), moved the 277 clinical specimens versus Ct value to the main text (figure 4) and substantially expanded the results section describing that result, and moved the pairwise phylogenetics into the main text (figure 5).

2. Certain areas of the 'Results' can be further elaborated and described. For example, lines 138-140 where it simply states "We performed 14 Nanopore sequencing runs to validate and characterize our method. A summary of these runs is available in the supplemental materials" is not sufficient for the 'Results' section paragraph and should really be elaborated given that these runs are important for the paper.

We thank the reviewer for this comment. We agree that the certain results sections can be clarified. We have expanded the sequencing runs results section to clarify the roles of these sequencing runs in validating the method.

3. The abstract states, "While it is adaptable to other sequencing platforms, the Nanopore platform validated here...". I would suggest making a separate figure and section in the 'Results' that compares performance of different platforms. If not, then this statement in the 'Abstract' is an overstatement and this manuscript should mainly be focused on Oxford Nanopore technologies, which has merit in itself as well. Please revise accordingly.

We thank the reviewer for this important comment. We agree that this may confuse the audience with regards to what was specifically validated and how it may be applied. To clarify this for the reviewer, nanopore sequencing was used for the validation and is the sequencing method routinely used. However, the main development here is the RT-PCR, not the sequencing method used, and other laboratories may use nearly any sequencer for the 2.2 and 2.5 kb amplicons. For simplicity, the final sentence of the abstract has been deleted.

4. Much of the 'Discussion' was written as a description for 'ongoing projects' and 'future steps' with this project, which is acceptable. However, some of the key components for a typical 'discussion' section appears to be missing. For example, how does your protocol compare to other assays ran on Oxford Nanopore? What are the limitations of this study?

We thank the reviewer for this important comment. We agree that the discussion should include caveats and limitations. To address this issue, we have added discussion regarding long amplicons and their relationship to sensitivity as well as the effect established WGS of SC2 has had on the rollout of this method.

Minor comments:

1. Some of the supplemental data has the Qiagen header in the data and it appears like these are raw printouts of the data. Is this allowed for publication since Qiagen's logo and trademark is being used? Please present the data in another way and make your own figures with the raw data.

QIAxcel data has been reformatted and the figures have been replaced.

Re: Spectrum02982-23R1 (Targeted Amplification and Genetic Sequencing of the Severe Acute Respiratory Syndrome Coronavirus 2 Surface Glycoprotein)

Dear Dr. Matthew W Keller:

Your manuscript has been accepted, and I am forwarding it to the ASM production staff for publication. Your paper will first be checked to make sure all elements meet the technical requirements. ASM staff will contact you if anything needs to be revised before copyediting and production can begin. Otherwise, you will be notified when your proofs are ready to be viewed.

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Thank you for submitting your paper to Spectrum.

Sincerely,
Day-Yu Chao
Editor
Microbiology Spectrum

Reviewer #1 (Comments for the Author):

The reviewer comments have been appropriately considered and addressed.

Reviewer #2 (Comments for the Author):

The authors have provided a response to my suggestions and included a text to highlight this in the main text.

Reviewer #3 (Comments for the Author):

Thank you for the revisions.