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Rapid Detection of SARS-CoV-2 Variants by Molecular Clamping Technology Based RT-qPCR

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Corresponding Author(s): Michael Sha, DiaCarta Inc

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Re: Spectrum04248-23 (Rapid Detection of SARS-CoV-2 Variants by Molecular Clamping Technology Based RT-qPCR)

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Thank you for the privilege of reviewing your work. Below you will find my comments, instructions from the Spectrum editorial office, and the reviewer comments.

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Sincerely,
Leiliang Zhang
Editor
Microbiology Spectrum

Reviewer #1 (Comments for the Author):

I find this study is interesting to read and has novel aspects despite several ambiguities.
Twist SARS-CoV-2 RNA controls 16, 17, 23 and 48 were used in the study, please describe which control corresponds to which VOC; why only 4 control VOCs were used and no control 14 of Alpha variant for LoD study? Should an ancestry virus of Hu-1 be included as non-variant control?

It is not clear how QuantiVirusTM Variants Detection Kit will be used since it consists of 3 tubes of reagents. Would the same samples be split into 3 reactions? Why authors cannot combine them into a single tube for multiplexing (or 2 tubes)? If this is one of the limitations of qRT-PCR multiplexing (up to 4 pairs), authors should include it in the discussion.
Authors claim this assay has lower cost, but I don't see any evidence. From my understanding this method is a bit more expensive than standard qRT-PCR since extra sets of PNA primers and/or probes are needed, or similar cost if no regular PCR primer/probe is needed. How much is the cost of PNA qRT-PCR per sample compared to qRT-PCR alone? How much does PNA synthesis cost, as I know special chemical synthesis is expensive. If not cheaper, I suggest removing those statements in the text.
Authors should include cost comparison analysis. If authors want to say this method is cheaper than viral WGS, they should state clearly; but I don't think it is a fair comparison with WGS in terms of cost, since WGS can resolve whole genome sequences of the virus and detect novel variants that authors' method cannot.
I believe this method is not faster than standard qRT-PCR, or the similar turn-around time; could authors comment on this since "rapid" word is used many times in the paper, but comparison of TAT is not mentioned at all. The time for each step in Fig. 2 is over-optimistic in my opinion, for example, hard to imagine 90 minutes for sample accession and transfer (from individual sample tube to wells of 2 plates) using biorobot plus extraction time (prepare reagents); not likely 150 minutes for amplifying 384 samples including time of reagent prep and dispensing. Is it feasible to make a clinical diagnosis of 384 samples in 10 minutes (data analysis)?
Minor issues:
Several citation issues: is reference 3 an online publication? Exact date of publication, page number and access date by author should be included. Same for reference 1 and 2. Reference 7 and 8 are not related to B.1.427/429, a key reference should be included. Deng X, Garcia-Knight MA et al. 2021. Transmission, infectivity, and neutralization of a spike L452R SARS-CoV-2 variant. Cell. Jun 24;184(13):3426-3437.e8.
Several pre-prints from medRxiv or BioRxiv (2018-2021) have published versions, authors should cite journal articles.

Authors should update variants in circulation, as I believe right now only omicron descendants are circulating such as HV.1, JN.1, XBB.

Authors should briefly review existing multiplexing RT-PCR methods for VOC detection, such as CoVarScan, LNA.

Line 140, The XNA we used in this study is a class of chemically-modified peptide nucleic acids with improve and more hydrophilic structure. Could authors discuss previous work of others using PNA for SARS-CoV-2 detection (give citations), what are differences of authors' approach from others? Such as these articles: https://pubmed.ncbi.nlm.nih.gov/36708624/, https://pubmed.ncbi.nlm.nih.gov/36228554/

Reviewer #2 (Comments for the Author):

Shen et al. applied the xenonucleic acid-based molecular-clamping technology to develop a multiplex RT-qPCR assay for SARS-CoV-2 multivariant detection.
The results showed that the assay was able to correctly identify all 36 Delta variant samples and all 34 Omicron samples. There are some issues in the manuscript that need to be addressed.
1. The samples used in the study are limited and their representativeness is also limited. Therefore, the results should be interpreted cautiously, the limitations should be pointed out, and directions and suggestions for future research should be proposed to gain a more comprehensive understanding of the local transmission of COVID-19 virus strains.
2. The positioning of the method discussed in the article is inaccurate. It cannot replace NGS, but provides another tool.
3. Adding comparisons and discussions with other rapid detection methods, such as High Resolution Melting (HRM), to provide a more comprehensive evaluation of the advantages and limitations of the assay.

RESPONSE TO REVIEWERS

Reviewer #1 (Comments for the Author):

R1-1. *I find this study is interesting to read and has novel aspects despite several ambiguities. Twist SARS-CoV-2 RNA controls 16, 17, 23 and 48 were used in the study, please describe which control corresponds to which VOC; why only 4 control VOCs were used and no control 14 of Alpha variant for LoD study? Should an ancestry virus of Hu-1 be included as non-variant control?*

Response:

In the methods section, we have included specific details about the VOCs associated with Twist SARS-CoV-2 RNA controls 16 (Beta), 17 (Gamma), 23 (Delta), and 48 (Omicron).

The aim of our sensitivity analysis was to establish the limit of detection (LoD) for each gene targeted by the mutations, rather than for the individual VOCs. Control 14 (Alpha) was excluded from the LoD study because it shares overlapping targets (D614G and N501Y) with control 16 (Beta).

The variant detection kit contains a Negative Control (NC), which serves as the wild-type control for all mutation targets and is considered as comparable to the Hu-1 non-variant control. Including the NC in each qPCR test ensures the absence of false positives. Consequently, an additional Hu-1 wild-type RNA control was not necessary due to wildtype control existing. This clarification has been added to the Methods section, under the Real-time reverse-transcription PCR subsection.

R1-2*. It is not clear how QuantiVirus™ Variants Detection Kit will be used since it consists of 3 tubes of reagents. Would the same samples be split into 3 reactions? Why authors cannot combine them into a single tube for multiplexing (or 2 tubes)? If this is one of the limitations of qRT-PCR multiplexing (up to 4 pairs), authors should include it in the discussion.*

Response

Thanks to the Reviewer pointing it out. Yes, qPCR has its assay limitation on 4 channels. Our QuantiVirus™ Variant Detection Kit is designed for practical use, and one sample is splited to three tubes. As seen in S. Table 1, there are multiple (3) multiplexing tubes included in this kit due to: i) more than 4 viral targets need to be detectable, but the instrument has 4 channels only, detection of which is maximally available for current PCR instruments; ii) other reasons using

multiple tubes is that minimization of assay interference by assigning too close targets like L452 and T478 to different tubes. For this assay shortage, we added this to the Discussion section for qPCR limitations.

R1-3. *Authors claim this assay has lower cost, but I don't see any evidence. From my understanding this method is a bit more expensive than standard qRT-PCR since extra sets of PNA primers and/or probes are needed, or similar cost if no regular PCR primer/probe is needed. How much is the cost of PNA qRT-PCR per sample compared to qRT-PCR alone? How much does PNA synthesis cost, as I know special chemical synthesis is expensive. If not cheaper, I suggest removing those statements in the text. Authors should include cost comparison analysis. If authors want to say this method is cheaper than viral WGS, they should state clearly; but I don't think it is a fair comparison with WGS in terms of cost, since WGS can resolve whole genome sequences of the virus and detect novel variants that authors' method cannot.*

Response

Thanks for the suggestion. In fact, Our QuantiVirus™ Variant Detection Kit is designed for practical use also in terms of the cost. We compared NGS target sequencing, but not WGS for its cost. For NGS target sequencing (identify couple of the variant), it costs $250-350 per sample. Our assay only costs $5-10 per sample. It is 50-fold cheaper. Compared to NGS method in existing SARS-CoV-2 variants detection, the cost (time, reagents, and other resources) using RT-PCR is considerably cheaper; but surely NGS cannot be replaced by PCR on finding / discovering the emerging unknown variants if WGS is used. Further, comparing to regular RT-qPCR, our PCR method using XNA molecular clamping technology offers more straightforward and easier interpretation and conclusion from experimental data, thus saving time and resources. The design and synthesis of our molecular clampers namely XNAs were optimized so as to merely add a small extra cost (lower than the cost of well-known Taqman probe).

R1-4*. I believe this method is not faster than standard qRT-PCR, or the similar turn-around time; could authors comment on this since "rapid" word is used many times in the paper, but comparison of TAT is not mentioned at all. The time for each step in Fig. 2 is over-optimistic in my opinion, for example, hard to imagine 90 minutes for sample accession and transfer (from individual sample tube to wells of 2 plates) using biorobot plus extraction time (prepare reagents); not likely 150 minutes for amplifying 384 samples including time of reagent prep and dispensing. Is it feasible to make a clinical diagnosis of 384 samples in 10 minutes (data analysis)?*

Response

For our working flow, during the sample aliquoting process, we utilized the MGI's MGISTP-7000 (a high-throughput automated sample transfer processing system) to transfer VTM to a 96-well plate, which was then processed in the MGISP-NE384 for viral RNA extraction. The MGISTP-7000 can handle the transfer of 192 samples (2 x 96 well plates) in **40 minutes**. we applied MGI instrument MGISP-NE384 which this instrument automatic handle and extracts 384 samples (4 x 96 well plates) in **20 min**. So, 90 min for 192 samples accession is doable.

Since our assay uses three tubes for one sample, each 384 qPCR plate can test a maximum of 125 samples of Variant test, along with test control PC/NC/NTC. The qPCR step requires **150 minutes**: 120 minutes for the qPCR run and an additional 30 minutes for loading samples and reagents onto the 384-well plates by multiple pipets.

Since we have developed software, the result can be automatic show up when the qPCR Ct is available for these 125 samples. All we need is to re-check its amplification curve if we have any questions or comments. Therefore, **10 min** is enough for data confirmation.

Minor issues:

R1-5*. Several citation issues: is reference 3 an online publication? Exact date of publication, page number and access date by author should be included. Same for reference 1 and 2. Reference 7 and 8 are not related to B.1.427/429, a key reference should be included. Deng X, Garcia-Knight MA et al. 2021. Transmission, infectivity, and neutralization of a spike L452R SARS-CoV-2 variant. Cell. Jun 24;184(13):3426-3437.e8.*
Several pre-prints from medRxiv or BioRxiv (2018-2021) have published versions, authors should cite journal articles.

Response

We revised and updated accordingly. For references related to Epsilon Variant (B. 1. 427/429), two literatures are now cited correctly including the one above. All preprints are now updated to their formal publications wherever applicable. For other references, updates (minor) are also made wherever applicable. Thanks for the reviewer's thoughtful check and suggestions.

R1-6. *Authors should update variants in circulation, as I believe right now only omicron descendants are circulating such as HV.1, JN.1, XBB.*

Response

In the Introduction section, we updated the variants in circulation accordingly based on the WHO, CDC and ECDC website. (June 10th, 2024)

R1-7. *Authors should briefly review existing multiplexing RT-PCR methods for VOC detection, such as CoVarScan, LNA.*

Response

We revised and enriched the comparisons across various RT-PCR methods for VOC detection in the Discussion section, including the CoVarScan and LNA.

R1-8. *Line 140, The XNA we used in this study is a class of chemically-modified peptide nucleic acids with improve and more hydrophilic structure. Could authors discuss previous work of others using PNA for SARS-CoV-2 detection (give citations), what are differences of authors' approach from others? Such as these articles: https://pubmed.ncbi.nlm.nih.gov/36708624/, https://pubmed.ncbi.nlm.nih.gov/36228554/*

Response

We added this accordingly in the Discussion section.

To our best knowledge, in contrast to the novel XNA-assisted RT-qPCR method for RNA detection of SARS-CoV-2 variants, there have been no studies reported to utilize the molecular clampers like peptide nucleic acids (PNAs) in SARS-CoV-2 variant detection and identification. One closest example was to use PNA clampers in digital PCR for variant detection reported most recently (64). Additionally, other studies using PNA clampers have developed a non-PCR methods, such as a biosensor technique (65) and Loop-Mediated Isothermal Amplification based for SARS-CoV-2 variants detection (66).

64. Zhang L, Parvin R, Lin S, Chen M, Zheng R, Fan Q, Ye F. 2024. Peptide Nucleic Acid Clamp-Assisted Photothermal Multiplexed Digital PCR for Identifying SARS-CoV-2 Variants of Concern. Advanced Science 11:2306088.

65. Li Y, Zhao S, Xu Z, Qiao X, Li M, Li Y, Luo X. 2023. Peptide nucleic acid and antifouling peptide based biosensor for the non-fouling detection of COVID-19 nucleic acid in saliva. Biosensors and Bioelectronics 225:115101.

66. Iijima T, Sakai J, Kanamori D, Ando S, Nomura T, Tisi L, Kilgore PE, Percy N, Kohase H, Hayakawa S, et al. 2023. A New Method to Detect Variants of SARS-CoV-2 Using Reverse

Transcription Loop-Mediated Isothermal Amplification Combined with a Bioluminescent Assay in Real Time (RT-LAMP-BART). International Journal of Molecular Sciences 24:10698.

Reviewer #2 (Comments for the Author):

Shen et al. applied the xenonucleic acid-based molecular-clamping technology to develop a multiplex RT-qPCR assay for SARS-CoV-2 multivariant detection. The results showed that the assay was able to correctly identify all 36 Delta variant samples and all 34 Omicron samples. There are some issues in the manuscript that need to be addressed.

***R2-1**. The samples used in the study are limited and their representativeness is also limited. Therefore, the results should be interpreted cautiously, the limitations should be pointed out, and directions and suggestions for future research should be proposed to gain a more comprehensive understanding of the local transmission of COVID-19 virus strains.*

Response

The clinical samples (649 in total) are relatively limited, while they had been collected in multiple short periods to quickly track the new cases. We added a paragraph in the Discussion section to point out the limitations and made suggestions for future directions.

***R2-2**. The positioning of the method discussed in the article is inaccurate. It cannot replace NGS, but provides another tool.*

Response

We have thought similarly to the reviewer in this concern, and made it clearer now (in both Introduction and Discussion sections). Molecular clamping qRT-PCR is not intended to replace the NGS but a rational supplementary tool in detecting and monitoring the new variants, and could be highly significant and useful for quickly screening the VOCs among a large number of samples in a lower cost.

***R2-3**. Adding comparisons and discussions with other rapid detection methods, such as High Resolution Melting (HRM), to provide a more comprehensive evaluation of the advantages and limitations of the assay.*

Response

Thanks for the suggestion. We further enriched the comparisons and discussions of various methods in the Discussion section accordingly.

Re: Spectrum04248-23R1 (Rapid Detection of SARS-CoV-2 Variants by Molecular Clamping Technology Based RT-qPCR)

Dear Dr. Michael Y. Sha:

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,
Leiliang Zhang
Editor
Microbiology Spectrum

Reviewer #2 (Comments for the Author):

All issues have been addressed.