nature biomedical engineering

PEER-REVIEW INFORMATION

**Ultrasensitive high-resolution profiling of early seroconversion in COVID-19 patients**

Corresponding author: David Walt

## Editorial note

This document includes relevant written communications between the manuscript's corresponding author and the editor and reviewers of the manuscript during peer review. It includes decision letters relaying any editorial points and peer-review reports, and the authors' replies to these (under 'Rebuttal' headings). The editorial decisions are signed by the manuscript's handling editor, yet the editorial team and ultimately the journal's Chief Editor share responsibility for all decisions.

Any relevant documents attached to the decision letters are referred to as **Appendix #**, and can be found appended to this document. Any information deemed confidential has been redacted or removed. Earlier versions of the manuscript are not published, yet the originally submitted version may be available as a preprint. Because of editorial edits and changes during peer review, the published title of the paper and the title mentioned in below correspondence may differ.

This manuscript was previously reviewed at a journal that is not offering transparent peer review. Peer-review information from that journal is therefore not included in this document. The reviewers chosen by the editor of this manuscript did not participate in the peer review of the work at the previous journal.

## Correspondence

Tue 07/07/2020
**Decision on Article nBME-20-1528-T**

Dear Dr Walt,

Thank you for your patience in waiting for the feedback on your Article, "Ultra-Sensitive High-Resolution Profiling of Anti-SARS-CoV-2 Antibodies for Detecting Early Seroconversion in COVID-19 Patients". As noted in previous communication, the manuscript has been seen by two experts, whose reports you will find at the end of this message. You will see that the reviewers have good words for the work, and that they raise a number of minor technical points that I am sure you will be able to address. In addition,

* Please clearly discuss the limitations of the assay, including the need for commercial hardware and reagents and the approximate cost of these.

* For purposes of replicability, reproducibility and data re-use, I strongly encourage you to deposit all the raw and analysed datasets (including the data underlying the figures and any other data supporting the findings) in a public repository. We recommend that you use a discipline-specific, community-recognized repository (a list of recommended repositories is available) or a generalist repository (such as figshare or Dryad). A citation to the deposited data can then be added at the end of the reference list of the manuscript, and the Data Availability statement should include phrasing such as 'Source data for the figures in this study are available in [repository name, e.g. figshare] with the identifier(s) [data DOI(s), e.g. doi:10.6084/m9.figshare.xxxxxxx] (ref. X).' The entry in the reference list would then read: Surname, N. et al. Dataset for [Title of the Article]. figshare https://doi.org/10.6084/m9.figshare.xxxxxxx (20XX).

* Please make the table in Fig. 4 a standalone table (Table 1). House style doesn't allow for tables in figures.

When you are ready to resubmit your manuscript, please upload the revised files, a point-by-point rebuttal to the comments from all reviewers, the (revised, if needed) reporting summary, and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

Please follow the following recommendations:

* Clearly highlight any amendments to the text and figures to help the reviewers and editors find and

understand the changes (yet keep in mind that excessive marking can hinder readability).

* If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).

* If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).

* Consider including responses to any criticisms raised by more than one reviewer at the beginning of the rebuttal, in a section addressed to all reviewers.

* The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers will the reports as they appear at the end of this message).

* Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We hope that you will find the referee reports helpful when revising the work, which we look forward to receive. Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

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Reviewer #1 (Report for the authors (Required)):

In their manuscript Norman and colleagues describe a sensitive assay to detect SARS-CoV-2 antibodies. The manuscript is interesting (although a little bit on the advertisement for a product site) but there are several points that need the authors' attention.

Major points

1) Many of the points made against ELISA on Page 3 are wrong. ELISAs have been shown to detect SARS-CoV-2 antibodies 2-3 days post onset of symptoms. Several ELISAs have been developed that are highly specific (see FDA EUAs). ELISAs can measure reactivity to multiple targets at the same time. And they are actually currently used to monitor the immune response over time. A more balanced discussion about this is needed. ELISAs can also be run in a rural lab in e.g. Bangladesh and don't require the purchase of a >100k piece of equipment. Please tone this down or make it balanced.

2) Please explain better what the full marker panel is and why its specificity is so low.

3) The negative control samples should be checked for reactivity to all 4 hCoVs.

4) It is unclear if the immunocompromised patients were excluded. If yes, why. A lot of COVID-19 patients are actually immunocompromised.

5) Was serum assessed as well.

6) The analysis looks good but the raw data shown in Figure 2 shows substantial overlap between prepandemic samples and day 0-7 samples and not just for immunocompromised individuals. This suggests that in the vast majority of cases in this time window positivity cannot be correctly detected.

Minor points

1) Throughout the manuscript including the abstract: Abbreviations are not defined.

2) Page 2, line 2: COVID-19 cannot infect anybody, it is the disease not the causative agent.

3) Page 2, line 7: Due to the fact that NAAT can be positive for weeks this does not mean much.

4) Page 2, line 12: As above, there is no COVID-19 seroconversion, there can only be SARS-CoV-2 seroconversion.

5) Page 3, line 3: This is not the correct definition of COVID-19.

6) Page 3, line 15/16: 'SARS-CoV-2', not 'COVID-19'

7) Page 4, lines 14 and 15; page 7, lines 10 and 11; page 13, line 2 and 20 etc.: Please use non-capitalized letters mid-sentence.

8) Page 5, line 20: Define 'CV'.

9) Page 10, line 6: 'herd immunity' is not used anymore. Use 'community immunity'.

10) How were NP and S1 produced?

11) Page 16, line 18: SARS-CoV-2 positive!

Reviewer #2 (Report for the authors (Required)):

This report comes from a fantastic group with excellent track record in ultrasensitive biosensing. Walt and co-workers have adapted their single-molecule array technology to develop an anti-SARS-CoV 2 antibody detection assay where the authors take advantage of the assay format for multiplexing to simultaneously investigate IgG, IgM and IgA binding interactions with four chosen SARS-CoV-2 targets. Through the assay's use of anti-human immunoglobulin antibodies, the authors observe higher levels of IgG, IgM and IgA against SARS-CoV-2 viral targets in NP RT-PCR-determined SARS-CoV 2 positive subjects when compared to a pre-pandemic group. The inclusion of individuals with a documented history of recent respiratory infection in the pre-pandemic group as well as comparison to symptomatic individuals determined to be SARS-CoV-2 negative provides good evidence for the insignificance of any potential non-specific binding from other immunoglobulins. A benefit of the assay to provide
dynamic quantification of the antibody-antigen binding interactions allows monitoring of immune response over the course of infection and the authors use the assay to platform four longitudinal case studies and provide data suggesting that the magnitude and antibody specificity of the immune response against SARS-CoV-2 may predict disease outcome.

The assay providing precise quantification of binding interactions over 4 orders of magnitude is desired as it appears that data points from the pre-pandemic group alone varies over 2-3 orders of magnitude. Optimised antigen-antibody subset data used in logistic regression models is used in a blinded samples (n=300) giving an impressive sensitivity and specificity of 99% (best case model example), though this data set does not include any samples collected within 4 days of a positive NP RT-PCR test. Detection of early seroconversion (0-6 days after a positive NP RT-PCR test) using the optimised subset data for NP RT-PCR-confirmed individuals in the training cohort gave a decent but inferior to NP RT-PCR sensitivity of 81% at 100% specificity. The assay requires the use of >1 µL of blood and the test itself is comparatively quick (ca. 20 mins preparation time not including wash time and plate reading). There is potential for the technique to act as a complement to current NP
RT-PCR techniques due to the detection of immunoglobulins as opposed to RNA and this is highlighted where the authors have reported the observation of significant antibody-antigen interaction in patients that were determined SARS-CoV-2 negative by NP RT-PCR, thus offering an approach to determine potential false negatives (highlighted in comparison of pre-pandemic data points to NP RT-PCR negative data points). Overall I liked this paper which is well written, well-presented and of course very timely. I have some suggestions that I hope the authors will find useful:

• Please include some discussion on the limitations of antibody testing (e.g. link between serology conversion and long term immunity still being understood and now the additional role that T cells might play). I don't think it detracts from the work to acknowledge these points more head on.

• Line 14 Page 4, should it be 4 viral epitopes? Instead of targets? S1 and RBD are parts of the spike

protein. So really its only 2 viral targets/proteins. Same on page 7 line 5.

• Supplementary tables 1-3: which antigens are used for these antibody pair testing?

• In supplementary figure 6, explain meaning of CV better.

• Supplementary table 6 represents the t-test analysis of Figure 2. Was there a test to confirm that there was normal distribution of the data to assume that t-test was the correct statistical analysis? It does not seem that the difference is that significant for all the test groups, "IgM, RBD, 0-3 days" seems not to have any visual difference with prepandemic control.

• Is there any statistical difference between the 1000 and 4000 dilution?

• If you have had chance to collect/analyse more patient samples since submitting the paper please consider including.

Tue 21/07/2020
**Reviewer reports for Article NBME-20-1528A**

Dear Dr Walt,

Thank you for your revised Article, "Ultra-Sensitive High-Resolution Profiling of Anti-SARS-CoV-2 Antibodies for Detecting Early Seroconversion in COVID-19 Patients", which has been seen by the two reviewers (see their comments below). Would you be able to run ELISA tests for any potential across-reactivity of the control (pre-pandemic) samples, as highly recommended by Reviewer #1? If so, I'll be happy to follow up with additional instructions for you to submit the final files for production.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

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Reviewer #1 (Report for the authors (Required)):

The authors responded well to many comments but there are still several points that need to be addressed:

1) The control sera need to be tested for reactivity to all 4 hCoVs. This is critical and needs to be performed. Reagents are commercially available, simple ELISAs would be enough. This is not a big ask and would confirm the authors' assumptions that these sera are actually positive.

2) Initial minor point 6: The prevalence of antibodies to SARS-CoV-2 is tested, not the prevalence of COVID-19. COVID-19 is the disease. A good proportion of individuals who have been infected with SARS-CoV-2 never had the actual disease and were asymptomatic.

3) Initial minor point 10: Well, then that should be easy. Just describe how they were made if the information is available. You want to spare the reader an investigation into Raybiotech's and Sino's CoAs.

Reviewer #2 (Report for the authors (Required)):

I’m happy with the response to reviewers and for this to now be accepted.

Tue 21/07/2020
**Re: Reviewer reports for Article NBME-20-1528A**

Dear Dr Pàmies,

Thank you for your email and continued interest in publishing our work.

Our control cohort was specifically selected to include patients diagnosed with a variety of respiratory diseases. We have detailed information about those patients including several who tested positive for other viral infections. We would be happy to add this information to the SI. No patients who had specifically tested positive for other human coronaviruses were available, as it is not common medical practice to sequence for the common cold.

Since we are not claiming that those samples cross react with all 4 hCoVs, we think the simplest and most expedient solution is to remove the following sentence from our discussion: 'Although we cannot comment on active non-SARS-CoV-2 coronoviridae infection nor previous exposure to such viruses among these individuals, such viruses are a common cause of respiratory tract infections, and thus are likely present among the 100 pre-pandemic respiratory infection cohort'.

However, if you think it is still necessary to run the experiments suggested by Reviewer 1 in order to be published in NBME, we will order the reagents and measure the samples. We believe that this will delay the submission by another 3-4 weeks due to reagent availability. We can do this work, but we think it is unnecessary as it doesn't change the results and it is more important to get this work out in a timely manner, as the review process is now nearing three months between Nature and NBME.

My sincere appreciation for your efforts.

Please advise,

David

Wed 22/07/2020
**Re: Reviewer reports for Article NBME-20-1528A**

Dear Dr Walt,

I think it is actually essential to your claim of having truly negative controls that the various cohorts of patients with documented respiratory infection do show antibodies to some of the other circulating coronaviruses.

Because it is highly likely that you will find that most of these sera are positive for endemic coronaviruses, and hence support your claims of no significant cross-reactivity with your chosen antigens for the assay, I would be happy to proceed with an 'accept in principle'. These additional data can be added to the supplementary information, so this file could be replaced at the latest when you return the proofs of the Article. Our production team needs at least two weeks (and typically it takes 35–40 days from Accept to publication), so these experiments and the accept and production processes could be run in parallel.

How does this sound?

Pep

Wed 22/07/2020
**Re: Reviewer reports for Article NBME-20-1528A**

Dear Dr Pàmies:

We have a deal. Thanks. Great working with you.

All the best,

David

Mon 03/08/2020
**Decision on Article NBME-20-1528A**

Dear Dr Walt,

Thank you again for your revised manuscript, "Ultra-Sensitive High-Resolution Profiling of Anti-SARS-CoV-2 Antibodies for Detecting Early Seroconversion in COVID-19 Patients". As noted in earlier communication, having consulted with Reviewers #1 and #2 (whose comments you will find at the end of this message), I am pleased to say that we shall be happy to publish the manuscript in *Nature Biomedical Engineering*, provided that the reactivity data from control sera for the four endemic coronaviruses are added to the manuscript and that the points specified in the attached instructions file are addressed.

When you are ready to submit the final version of your manuscript, please upload the files specified in the instructions file.

For primary research originally submitted after December 1, 2019, we encourage authors to take up transparent peer review. If you are eligible and opt in to transparent peer review, we will publish, as a single supplementary file, all the reviewer comments for all the versions of the manuscript, your rebuttal letters, and the editorial decision letters. **If you opt in to transparent peer review, in the attached file please tick the box 'I wish to participate in transparent peer review'; if you prefer not to, please tick 'I do NOT wish to participate in transparent peer review'.** In the interest of confidentiality, we allow redactions to the rebuttal letters and to the reviewer comments. If you are concerned about the release of confidential data, please indicate what specific information you would like to have removed; we cannot incorporate redactions for any other reasons. If any reviewers have signed their comments to authors, or if any reviewers explicitly agree to release their name, we will include the names in the peer-review supplementary file. More information on transparent peer review is available.

In addition, because one or more authors belong to institutions (MIT and Harvard FAS) with an open-access policy that is incompatible with our LTP, we have to ask you to collect and collate the necessary open-access waivers granted by the institution of every affected author (to obtain a waiver, some institutions offer an automated web form or a specific email address). The waivers should be uploaded with the manuscript files.

Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

**Response to reviews: Ultra-Sensitive High-Resolution Profiling of Anti-SARS-CoV-2 Antibodies for Detecting Early Seroconversion in COVID-19 Patients**

We would like to sincerely thank the reviewers for their helpful and constructive comments. We made the necessary revisions to the manuscript based on the reviewer's comments. We addressed all the comments laid out by the reviewers in addition to the editorial requests. We believe that these changes have further strengthened the manuscript.

**Editor comments:**

* Please clearly discuss the limitations of the assay, including the need for commercial hardware and reagents and the approximate cost of these.

Thank you for this comment. We chose not to include the cost of the test (~$20) as it is comparable to other antibody tests currently on the market for COVID19 serology. We have, however, added the following text to the manuscript to more clearly discuss the limitations of serological testing and our assay specifically:

1) While antibody testing is clearly key in curbing the COVID-19 pandemic, several limitations of antibody-based testing should be considered[25]. First, serological testing cannot replace NAATs for diagnosis of COVID-19 due to the later onset of seroconversion. Second, until a concrete link between levels of anti-SARS-CoV-2 antibodies and immunity from reinfection can be established, it is not possible to make medical decisions or to determine matters of public health based on this type of testing. Finally, even once a link between antibody levels and immunity can be established, it will need to be determined how long this immunity lasts for proper clinical decision-making.
2) Although our Simoa serological assay is a powerful analytical tool to understand the host immune response of the COVID-19 pandemic, it requires a specialized instrument that is not widely available.

* For purposes of replicability, reproducibility and data re-use, I strongly encourage you to deposit all the raw and analysed datasets (including the data underlying the figures and any other data supporting the findings) in a public repository. We recommend that you use a discipline-specific, community-recognized repository (a list of recommended repositories is available) or a generalist repository (such as figshare or Dryad). A citation to the deposited data can then be added at the end of the reference list of the manuscript, and the Data Availability statement should include phrasing such as "Source data for the figures in this study are available in [repository name, e.g. figshare] with the identifier(s) [data DOI(s), e.g. doi:10.6084/m9.figshare.xxxxxxx] (ref. X)." The entry in the reference list would then read: Surname, N. et al. Dataset for [Title of the Article]. figshare https://doi.org/10.6084/m9.figshare.xxxxxxx (20XX).

Data was uploaded to Mendeley Data and a data availability statement was added to the paper.

* Please make the table in Fig. 4 a standalone table (Table 1). House style doesn't allow for tables in figures.

| |
|---|
| Figure 4 was corrected and Table 1 was added |

**Reviewer #1**

In their manuscript Norman and colleagues describe a sensitive assay to detect SARS-CoV-2 antibodies. The manuscript is interesting (although a little bit on the advertisement for a product site) but there are several points that need the authors' attention.

Major points

1) Many of the points made against ELISA on Page 3 are wrong. ELISAs have been shown to detect SARS-CoV-2 antibodies 2-3 days post onset of symptoms. Several ELISAs have been developed that are highly specific (see FDA EUAs). ELISAs can measure reactivity to multiple targets at the same time. And they are actually currently used to monitor the immune response over time. A more balanced discussion about this is needed. ELISAs can also be run in a rural lab in e.g. Bangladesh and don't require the purchase of a >100k piece of equipment. Please tone this down or make it balanced.

| |
|---|
| We thank the reviewer for pointing this out and have removed the following text from the paper:<br><br>Third, conventional ELISA can only analyze one type of immunoglobulin for a specific target at a time, limiting their ability to profile the underlying immune response.<br><br>We have also modified the text to indicate that the remaining points do not apply to all ELISA assays. We left the other points about low specificity and late detection of antibodies in because they remain true for the vast majority of SARS-CoV-2 ELISA serological assays currently on the market. |

2) Please explain better what the full marker panel is and why its specificity is so low.

The full marker model is a logistic regression model in which all the 12 interactions are included for training the model (this is also described in the text). It appears that the full marker panel model is pushing patients predicted probabilities higher than they are under the other models. This is helpful with a few cases who, under the early and late models, were incorrectly classified. This is why the Sensitivity is higher. Unfortunately, pushing patients predicted probabilities higher is harmful when it comes to Specificity.

No change was made to the manuscript because these points are implicit in the analysis.

3) The negative control samples should be checked for reactivity to all 4 hCoVs.

Unfortunately, we do not have this specific information but the pre-pandemic samples were run explicitly to test for such cross reactivity. This is why we state in the discussion section: 'Although we cannot comment on active non-SARS-CoV-2 coronoviridae infection nor previous exposure to such viruses among these individuals, such viruses are a common cause of respiratory tract infections, and thus are likely present among the 100 pre-pandemic respiratory infection cohort'.

4) It is unclear if the immunocompromised patients were excluded. If yes, why. A lot of COVID-19 patients are actually immunocompromised.

As written in the text and based on our findings in the discovery cohort (in which some immunocompromised patients did not seroconvert at late stages of disease), we chose to exclude immunocompromised individuals when building and training our seroconversion classification model. However, immunocompromised patients were NOT excluded from the blinded validation cohort. Interestingly the only false negative in this cohort for the early stage model was immunocompromised.

5) Was serum assessed as well.

We assessed the use of serum with our assay and obtained comparable results. However, since all the samples used for the three cohorts described in this paper were plasma samples, we chose not to include this data in this publication and do not think it is necessary to mention.

6) The analysis looks good but the raw data shown in Figure 2 shows substantial overlap between prepandemic samples and day 0-7 samples and not just for immunocompromised individuals. This suggests that in the vast majority of cases in this time window positivity cannot be correctly detected.

It is true that when looking at a single marker there is an overlap between early stage samples and the pre pandemic controls. However, the combination of several markers allows for better discrimination between the groups. This is the reason we chose to down select a combination of markers for our models. We show that the early stage model and the full marker models allow for >80% sensitivity in seroconversion classification of the early stage cases. As written in the text: 'the lower sensitivity during the first week undoubtedly stems from a delayed immune response in some individuals'.

Minor points

1) Throughout the manuscript including the abstract: Abbreviations are not defined.

Corrected throughout the text

2) Page 2, line 2: COVID-19 cannot infect anybody, it is the disease not the causative agent.

Thank you for this important comment. We corrected it here and throughout the text including the figures.

3) Page 2, line 7: Due to the fact that NAAT can be positive for weeks this does not mean much.

Corrected to: the first positive nasopharyngeal RT-PCR test after symptom onset

While it is true that it is not an objective indicator of time since the onset of infection, there can also be variability in the time between infection and symptom onset. Therefore, this is the only objective way we can indicate the sensitivity of our assay.

4) Page 2, line 12: As above, there is no COVID-19 seroconversion, there can only be SARS-CoV-2 seroconversion.

COVID-19 seroconversion was replaced with SARS-CoV-2 seroconversion

5) Page 3, line 3: This is not the correct definition of COVID-19.

Corrected to: Coronavirus disease 2019 (COVID-19)

6) Page 3, line 15/16: 'SARS-CoV-2', not 'COVID-19'

We disagree with this comment. Here we describe the prevalence of the disease (COVID19) and not the virus (SARS-CoV-2).

7) Page 4, lines 14 and 15; page 7, lines 10 and 11; page 13, line 2 and 20 etc.: Please use non-capitalized letters mid-sentence.

Corrected throughout the text

8) Page 5, line 20: Define 'CV'.

Corrected: coefficient of variation (CV)

9) Page 10, line 6: 'herd immunity' is not used anymore. Use 'community immunity'.

Corrected

10) How were NP and S1 produced?

This information is provided in the methods section: 'The nucleocapsid and S1 antigens were sourced commercially (Nucleocapsid: Ray Biotech 230-30164 and S1: Sino Biological V0591-V08H)'.

11) Page 16, line 18: SARS-CoV-2 positive!

Corrected

**Reviewer #2**

This report comes from a fantastic group with excellent track record in ultrasensitive biosensing. Walt and co-workers have adapted their single-molecule array technology to develop an anti-SARS-CoV 2 antibody detection assay where the authors take advantage of the assay format for multiplexing to simultaneously investigate IgG, IgM and IgA binding interactions with four chosen SARS-CoV-2 targets. Through the assay's use of anti-human immunoglobulin antibodies, the authors observe higher levels of IgG, IgM and IgA against SARS-CoV-2 viral targets in NP RT-PCR-determined SARS-CoV 2 positive subjects when compared to a pre-pandemic group. The inclusion of individuals with a documented history of recent respiratory infection in the pre-pandemic group as well as comparison to symptomatic individuals determined to be SARS-CoV-2 negative provides good evidence for the insignificance of any potential non-specific binding from other immunoglobulins. A benefit of the assay to provide
dynamic quantification of the antibody-antigen binding interactions allows monitoring of immune response over the course of infection and the authors use the assay to platform four longitudinal case studies and provide data suggesting that the magnitude and antibody specificity of the immune response against SARS-CoV-2 may predict disease outcome.

The assay providing precise quantification of binding interactions over 4 orders of magnitude is desired as it appears that data points from the pre-pandemic group alone varies over 2-3 orders of magnitude. Optimised antigen-antibody subset data used in logistic regression models is used

in a blinded samples (n=300) giving an impressive sensitivity and specificity of 99% (best case model example), though this data set does not include any samples collected within 4 days of a positive NP RT-PCR test. Detection of early seroconversion (0-6 days after a positive NP RT-PCR test) using the optimised subset data for NP RT-PCR-confirmed individuals in the training cohort gave a decent but inferior to NP RT-PCR sensitivity of 81% at 100% specificity. The assay requires the use of >1 µL of blood and the test itself is comparatively quick (ca. 20 mins preparation time not including wash time and plate reading). There is potential for the technique to act as a complement to current NP
RT-PCR techniques due to the detection of immunoglobulins as opposed to RNA and this is highlighted where the authors have reported the observation of significant antibody-antigen interaction in patients that were determined SARS-CoV-2 negative by NP RT-PCR, thus offering an approach to determine potential false negatives (highlighted in comparison of pre-pandemic data points to NP RT-PCR negative data points).
Overall I liked this paper which is well written, well-presented and of course very timely. I have some suggestions that I hope the authors will find useful:

• Please include some discussion on the limitations of antibody testing (e.g. link between serology conversion and long term immunity still being understood and now the additional role that T cells might play). I don't think it detracts from the work to acknowledge these points more head on.

Based on this comment and the editor's comment we added the following text to the discussion: 'While antibody testing is clearly key in curbing the COVID-19 pandemic, several limitations of antibody-based testing should be considered[25]. First, serological testing cannot replace NAATs for diagnosis of COVID-19 due to the later onset of seroconversion. Second, until a concrete link between levels of anti-SARS-CoV-2 antibodies and immunity from reinfection can be established, it is not possible to make medical decisions or to determine matters of public health based on this type of testing. Finally, even once a link between antibody levels and immunity can be established, it will need to be determined how long this immunity lasts for proper clinical decision-making.'

We also added a reference to support this text.

Furthermore, we include this content in the discussion section by stating a few questions that should be explored: 'In future studies, we aim to utilize this new method to address important unanswered questions such as which specific antigen-antibody interactions are important and how they relate to long-term immunity, how long these antibodies remain in the bloodstream, and how community immunity can affect the spread of COVID-19.'

• Line 14 Page 4, should it be 4 viral epitopes? Instead of targets? S1 and RBD are parts of the spike protein. So really its only 2 viral targets/proteins. Same on page 7 line 5.

We agree that it is not accurate to write 4 viral proteins. We therefore replaced proteins with targets throughout the text. We believe that 'target' is a better term than 'epitope' since each different targets may include several different epitopes for SARS-CoV-2 antibodies.

• Supplementary tables 1-3: which antigens are used for these antibody pair testing?

We agree that this section was not explained clearly in the SI. We made the following changes:

1. Catalogue numbers for the protein standard (antigen) used in IgM and IgA screening were added.
2. We clarified the text with regard to screening for IgM and IgA: 'In order to identify the highest affinity anti-IgM and anti-IgA antibodies, we screened candidates using a sandwich Simoa immunoassay for detection of the given immunoglobulin. In this screening process commercially available human-plasma-derived immunoglobulins (IgM Sigma-Aldrich I8260 and IgA Sigma-Aldrich I4036) were used as protein standards in a sandwich Simoa assay. We then cross-tested each anti-IgM or anti-IgA antibody as a capture antibody against all other candidate IgM or IgA antibodies as detectors in order to establish high binding affinity on the Simoa platform. This was done separately for IgM and IgA. Tight binders were selected for further analysis. Below, are tables indicating signal to noise ratios (SNR) for each antibody pair.'
3. We clarified the language for screening IgG antibodies: 'we screened anti-IgG detector antibodies using a mixture of three commercially available recombinant human IgGs to S1 (Creative Biolabs CR3022, CBFYR-0119, CBFYR-0120) and the four viral epitopes coated beads (spike, S1, RBD and nucleocapsid).'

• In supplementary figure 6, explain meaning of CV better.

Text was corrected: 'Coefficient of Variance (CV) of the duplicate measurements for each of the samples in the validation cohort. Each dot represents the CV of duplicate measurements for each of the 472 datapoints show in Figure 2. The red line indicates the median of the CVs. As can be seen all CVs were in an acceptable range indicating the accuracy of the measurements'.

• Supplementary table 6 represents the t-test analysis of Figure 2. Was there a test to confirm that there was normal distribution of the data to assume that t-test was the correct statistical analysis? It does not seem that the difference is that significant for all the test groups, "IgM, RBD, 0-3 days" seems not to have any visual difference with prepandemic control.

The data are not normally distributed, and comes from independent samples, and therefore a Mann-Whitney U test was performed. The IgM RBD indeed results in the poorest difference between cases and controls with a p value of 0.01.

For clarity we replaced the word ‘t-test’ with ‘Mann-Whitney U test’ in the text and SI.

• Is there any statistical difference between the 1000 and 4000 dilution?

There was no significant statistical difference between 1000 and 4000 dilution in terms of early seroconversion classification. However, the ability to use the 4000X diluted sample significantly increased the dynamic range of the assay, allowing quantification of early stage cases as well as late stage cases for which the signal was saturated in the 1000X dilution. Furthermore, the ability to dilute the sample by 4000X allows the use of less than 1 ul of sample and has a higher potential to prevent non-specific binding.

• If you have had chance to collect/analyse more patient samples since submitting the paper please consider including.

This paper now contains results from more than 800 samples. We believe that this large sample size is sufficient for demonstrating the advantages and potential use of our ultra-sensitive multiplex assay for early seroconversion classification and monitoring the immune response over time. Future studies will include data from additional sample cohorts in order to address additional scientific questions.

**Response to reviews: Ultra-Sensitive High-Resolution Profiling of Anti-SARS-CoV-2 Antibodies for Detecting Early Seroconversion in COVID-19 Patients**

**Reviewer #1:**

The authors responded well to many comments but there are still several points that need to be addressed:

1) The control sera need to be tested for reactivity to all 4 hCoVs. This is critical and needs to be performed. Reagents are commercially available, simple ELISAs would be enough. This is not a big ask and would confirm the authors' assumptions that these sera are actually positive.

We tested the reactivity of the control samples to all 4 hCoVs. The data was added to the SI. We also added the following text to the discussion:
Although our pre-pandemic control samples were not assessed for other coronavirus infections at the time of their illness, we assessed them for antibodies to the four common human coronaviruses and observed that several samples had particularly high levels for one or more of these species (Supplementary Figure 7).

2) Initial minor point 6: The prevalence of antibodies to SARS-CoV-2 is tested, not the prevalence of COVID-19. COVID-19 is the disease. A good proportion of individuals who have been infected with SARS-CoV-2 never had the actual disease and were asymptomatic.

Corrected to prevalence of antibodies to SARS-CoV-2

3) Initial minor point 10: Well, then that should be easy. Just describe how they were made if the information is available. You want to spare the reader an investigation into Raybiotech's and Sino's CoAs.

The information was added:
Recombinant SARS-CoV-2 Nucleocapsid protein with C-terminal His-tag was purchased from Ray Biotech (230-30164). It was derived from the transfected human HEK293 cells. SARS-CoV-2 S1 protein with C-terminal His-tag was purchased from Sino Biological (V0591-V08H). A DNA sequence encoding the S1 Subunit (Val16-Arg685) was expressed in HEK293 Cells.

**Reviewer #2:**

I'm happy with the response to reviewers and for this to now be accepted.