Response to comments

Please find response after each corresponding comment in color.

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| **Editorial comments:**   * ***NOTE: Please download this version of the Microsoft word document (File name: 54897\_R1\_060816) for any subsequent changes. Please keep in mind that some editorial changes have been made prior to peer review.***      * Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.   •Formatting: -Please define all abbreviations at first occurrence (such as RDE, etc.).  RDE and VGM have been defined.  -Please include spaces between numbers and units.  All have corrected.  -All figure legends should have a title and a brief description.  Brief descriptions were added to legends of Figure 1 and Figure 4.  -3.2 note – This should appear somewhere in section 2, not section 3.  It becomes NOTE 1.1 now.  •Grammar: -Please use American English throughout the manuscript. For instance, “optimisation" and “neutralisation" should be “optimization” and “neutralization”, respectively.  All have been corrected.  -Should be 96-well plates, not 96 well-plates.  All have been corrected.  -1.2 – “sterile the multiwall-plate washer”  Corrected.  -4.3.4 – “and Click”  Corrected.  •Visualization: Protocol is discontinuous. Steps 1.3 and 1.12-1.14 should also be highlighted for filming.  They are now highlighted.  •Additional detail is required: -What BSL level is required?  BSL level is virus dependent. Seasonal influenza viruses require BSL 2 level and potential pandemic influenza viruses require BSL 3+. An explanation has been added as NOTE 1.1.  -1.4 note – This note appears out of place. What manifold is being referred to?  It has been deleted.  -1.7 – What overlay? How is it prepared?  Overlay is explained in Materials and Equipment Table. It is made of 5ml 2X DMEM (Gibco 21935-028), Trypsin (Sigma T1426) (2μg/ml final conc) – 20μl of 1mg/ml stock, and  Avicell - 5ml.  -1.11 – What is PBS A?  Nature pH phosphate-buffered saline with following details: NaCl -10 gm, KCl - 0.25 gm, Na2HPO4 - 1.437 gm, KH2PO4 – 0.25 .gm, and Dist. Water – 1 L. It is in Materials and Equipment Table now.  -1.16, 1.18 – Which antibody is used? What is the dilution? What is it diluted in?  Mouse MAb against influenza type A (CDC –WHO kit used at 1:2000 in ELISA Buffer or Bio-Rad MCA 400 at 1:1000 in ELISA Buffer). It can be found in Materials and Equipment Table.  -1.17 – What is the composition of the wash buffer?  0.05% Tween – 80 in PBS A (v/v). It can be found in Materials and Equipment Table.  •Branding should be removed: -Line 231 – LabView  It has been removed.  -Please remove trademark symbols from the materials table.  They have been removed.  •Results: Please indicate what the results of the neutralization mean. How should this be interpreted?   The following sentences have been added in Representative Results to explain Figure 5 and Figure 6.  Titration results are illustrated in Figure 5. Figure 5d demonstrated the decrease of ICPs with the increase of virus dilution. The curves were normalized against the ICPs of the same viruses that yielded infection of all cells within a well12 (defined as ICP saturation). If the ICP did not reach saturation with the highest virus concentration, the ICP average from corresponding duplicates was used instead (A/Moscow/103/2015 in Figure 5d). The virus dilutions that produced 30% of ICP saturation were chosen as the input virus dilution for neutralization (Table 3).  And  Figure 6d showed the infection progress with the increase of serum dilution. Normalized Positive Population on vertical axis represents the ratio of ICPs from corresponding antisera responses against the average ICP of the reference virus12. The background ICP from uninfected cell controls was subtracted during the normalization. The neutralization titres were determined as the reciprocals of the antiserum dilutions corresponding to 50% ICP reduction in ICP (Table 4). Linear interpolation was used to estimate titres falling between two adjacent serum dilutions.   * If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.   A copy of the re-print permission has been attached during the revision.   * JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when Safiyaciting directly from PubMed. In these cases, please manually include DOIs in reference information.   All DOIs are added except papers published before 2000 by Journal of Clinical Microbiology and Reference 9. DOI cannot be found in these publications.  IMPORTANT: Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.              It is done.   * NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.   **Reviewers' comments:**  **Reviewer #1:** *Manuscript Summary:* This paper describes an imaging based micro neutralisation assay for use in analysing the antigenic relationship of influenza viruses. The paper is well written and provides a good overview of the method, which would be useful in characterising new influenza viruses.  *Major Concerns:* N/A  *Minor Concerns:* I have the following minor comments which need to be addressed. Line 57-58 i believe this should say for quantification of neutralising antibodies  It has been changed to: …for quantitation of neutralising antibodies and antiviral activities.  line 109 define ICP  It has been defined at its first appearance.  line 147 please explain why 6 or 5 tubes are used  The sentence has been changed to: Add 900 μl VGM to each of 6 sterile tubes (or less depending on the number of test viruses and duplicates).  line 156 how is the period of time optimised? Explain why 2-3 hours are used.  NOTE 1.7 is added to explain the optimised time.  Line 300 please include the subtype of the viruses in the representative results  Virus subtype has been added.  Figure 3 the set up panel is very difficult to read and should be larger, perhaps reducing the size of the plate  Figure 3 has been replaced by a higher resolution image.  line 311 why is A/Stockholm shown when other viruses have been used as test viruses?  Titration and neutralization results shown in Representative results were not from the same experiment. They are just used as examples to demonstrate the protocol.  line 311-12 Include the viruses the ferret antisera are specific for  The sentence has changed to: The reference virus shown is H3N2 A/Stockholm/63/2015.  line 358 please rewrite this sentence Therefore, the assay, ... to ensure it is understood by the reader  The sentence has been rewritten to: Therefore, the assay is capable to examine more viruses and the effects of a wider range of antibodies than HI, and helps to reflect more comprehensively the antigenic similarities or differences between viruses12.  line 379 Accurate measurements.... please check this sentence which is difficult to read  The sentence has been rewritten to: During a neutralization, antiserum response to an infection is normalized against the reference virus (VC) subtracted by the background level (CC). Accurate measurements of VC and CC are essential to a neutralization experiment.  *Additional Comments to Authors:* N/A   **Reviewer #2:** *Manuscript Summary:* Clearly presented, easy to read  *Major Concerns:* N/A  *Minor Concerns:* N/A  *Additional Comments to Authors:* N/A   **Reviewer #3:** *Manuscript Summary:* General comment This paper describes an image-based micro-neutralisation assay to quantify the true antigenic relationships or differences between influenza viruses. For decades, identification of antigenetic variants has been determined largely by the hemagglutination inhibition assay. However, interpretation of HI results has become complicated due to changes in receptor binding properties or selection of variants during isolation and passaging of viruses. Thus, microneutralisation (MN) is a valuable tool in complementing the HI assay. The setup and/or readout of the MN assays widely used is too time consuming for the large number of viruses to be analysed. Therefore, the MN assay including an automated quantitation described here may be of special interest for laboratories analysing the antigenic features of influenza viruses. The method presented here is, in general, identical to that published by Lin et al (Influenza and Other Respiratory Viruses, 2015). Further comments are listed below.  General points \*The authors wrote that an imaging-based MN assay was optimised in this study. \*However, the parameters and conclusions mentioned here were already described in the a.m. Lin et al., paper from 2015, e.g. as -Selection of the most suitable cell line -Optimisation of experimental design -Optimisation of data quantitation \*This fact raises the following question: Are there any new developments compared to the earlier published Lin paper? - If yes, they have to be clearly addressed - If no, this should be stated and the objective of this paper should be clear. The objective is, as far as I'm understand, to publish in this journal in order to allow interested scientists an easy access to this method provided by video support detailed protocols  The referee is absolutely correct. This manuscript is focused on the details of the protocol that have been developed in last two publications (please referring 11 and 12 in Reference).  Protocol \*Some abbreviations are not explained: VGM, RDE  The have been defined at their first appearance.  \*The Avicel overlay is a very import step. However, this is not included in the Excel sheet  It is in the Material and Equipment Table.  \*PBS A is missing in the Excel sheet. Moreover, the sheet seems to be incomplete with regard to all the reagents needed.  All reagents are in the Material and Equipment Table.  \*Point 2.4: No Supplementary was included in the original submission! What kind of details will be included in the Supplement?  Supplementary was included in the original submission. It is the instruction manual of the software. I will make sure that it is included in the submission.  \*Point 2.4: Figure 3 has fields that are differently coloured but this is not explained in the protocol.    Figure3 has been changed for higher resolution. Due to the length limitation of the manuscript and the focus of the protocol, the software is explained in Supplementary.  \*Please provide at least some general information regarding the software  A step-by-step explanation is given in Supplementary.  \*Please check the format of the Excel sheet! There are 12 lines, but there are discrepancies between the comments column and the column with the name of reagents (e.g. DMEM and overlay part 1)  It is all in line now.  Figures/Tables \*Very useful \*Table 3: The slash is missing after A/HK/………  It has been corrected.  *Major Concerns:* N/A  *Minor Concerns:* N/A  *Additional Comments to Authors:* N/A |