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Optimisation of a quantitative micro-neutralisation assay

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Abstract:	The plaque reduction assay is a standard technique for measuring influenza virus infectivity and inhibition of virus replication. In this study, we have optimised an imaging-based micro-neutralisation (MN) assay to quantify the true antigenic relationships between viruses, so as to confirm the emergence of new antigenic variants. The improvements include the selection of the most suitable cell line according to virus type or subtype, optimisation of the experimental design, well-plate imaging using flatbed scanner, and data quantitation with infective cell population. Our study demonstrates that the improved MN assay works well with influenza A(H1N1)pdm09, A(H3N2) and B viruses without significantly influenced by amino acid substitutions in the neuraminidase (NA) of A(H3N2) viruses, and is particularly useful for characterisation of viruses which either grow to low HA titre and/or undergo an abortive infection resulting in an inability to form plaques in cultured cells. In comparison to other available techniques, this approach is cost-effective, relatively accurate, reproducible and readily available.
Author Comments:	I would like Yipu Lin to be the first author as indicated in the submitted manuscript. Thanks!
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TITLE:
Optimization of a quantitative micro-neutralization assay

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SHORT ABSTRACT:
This study describes an imaging-based micro-neutralization assay to analyze the antigenic relationships between viruses. The protocol employs a flatbed scanner and has four steps, including titration, titration quantitation, neutralization, and neutralization quantitation. The assay works well with current circulating influenza A(H1N1)pdm09, A(H3N2), and B viruses.

LONG ABSTRACT:
The micro-neutralization (MN) assay is a standard technique for measuring the infectivity of the influenza virus and the inhibition of virus replication. In this study, we present the protocol of an imaging-based MN assay to quantify the true antigenic relationships between viruses. Unlike typical plaque reduction assays that rely on visible plaques, this assay quantitates the entire infected cell population of each well. The protocol matches the virus type or subtype with the selection of cell lines to achieve maximum infectivity, which enhances sample contrast during imaging and image processing. The introduction of quantitative titration defines the amount of input viruses of neutralization and enables the results from different experiments to be comparable. The imaging setup with a flatbed scanner and free downloadable software makes the approach high throughput, cost effective, user friendly, and easy to deploy in most laboratories. Our study demonstrates that the improved MN assay works well with the current circulating influenza A(H1N1)pdm09, A(H3N2), and B viruses, without being significantly influenced by amino acid substitutions in the neuraminidase (NA) of A(H3N2) viruses. It is particularly useful for the characterization of viruses that either grow to low HA titer and/or undergo an abortive infection resulting in an inability to form plaques in cultured cells.

INTRODUCTION:
Micro-neutralization (MN) assays are used in virology for the quantitation of neutralizing antibodies and of antiviral activities. As an alternative of hemagglutination inhibition (HI) assays, MN assays can overcome non-antigenic effects influenced by the affinity changes of receptor-binding in influenza viruses, which can complicate the interpretation of HI results[1,2,3]. Until recently, most MN assays were based on cytopathic effects (CPE) or enzyme-linked immunosorbent

assays (ELISA)[4, 5]. MN assays based on focus and plaque reduction were developed in 1990[6, 7, 8]. Plaque reduction assays rely on counting visible plaques to quantify infectivity. However, visual counts only cover large plaques that are resolvable by human eyes, even though the majority of plaques are small and invisible for many current circulating viruses. This incomplete coverage can cause significant variation among examiners and between experiments, leading to incomparable results. It is also impossible to use the method when some viruses show abortive infection of single cells or very small plaques.

The poor counting resolution can be improved by the introduction of an imaging-based protocol. With advances in technology, optical microscopy and high-throughput well-plate readers can provide accurate means to count the infected cells[9]. Under a trans-illuminated microscope, infected cells tagged with certain markers may be visualized by their absorption or fluorescence contrast in sub-cellular resolution. A sample can then be analyzed on a computer screen.

Unfortunately, due to the limitation in the field of view, more than a hundred tiled images are required to cover a single well. Analyzing a plate with 96 wells would require the imaging and processing of about ten thousand images. Such a laborious process is time consuming and expensive, and the resolution gained is in general unnecessary for routine characterization of viral infections. Laboratories with a limited budget may find that the approach built around a flatbed scanner provides a cost-effective, high-throughput alternative.

In this paper, we describe an improved plaque reduction MN assay that is suitable for the antigenic characterization of a large number of viruses and for quantitatively measuring antiviral activities and neutralization antibodies. The assay has several advantages: firstly, it is an imaging-based assay that is able to measure virus infections on the cellular level, regardless of plaque size. Counting the total infected cell population (ICP) within a well greatly increases the detection sensitivity, making it possible to characterize the viruses with low infectivity. Secondly, a more accurate quantitative titration is introduced prior to neutralization to determine the amount of input virus. The quantitative input virus significantly reduces the variation between different experiments and makes the results more comparable between laboratories. Thirdly, neutralization titers can be determined directly by analyzing images, making the quantitation fast and user-friendly. Finally, the protocol provides a cost-effective and high-throughput alternative with the required resolution and accuracy. The quantitation is based on a flatbed scanner and free data processing software. The whole setup has a small footprint and is deployable in most laboratories.

The protocol presented in this paper consists of four major steps, including virus titration, titration quantitation, virus neutralization, and neutralization quantitation. Virus titration is a preparation experiment that determines the amount of input viruses to be used in neutralization. During a titration, a number of viral concentrations are applied to the cell monolayers in a 96-well plate. The infected cells are then quantitated in **section** 2. The viral dilution that produces 20%-85% ICP is in turn applied as input viruses to the corresponding neutralization in section 3. The titers of the neutralization protocol are quantified using section 4. Experiments that followed the above protocols are presented in the Representative Results. The

assay has been tested thoroughly during the last two years with most of the current circulating influenza viruses, such as A(H1N1)pdm09, A(H3N2), and B viruses. The results of the influenza virus characterization were included in the reports for the WHO consultation meeting that gave recommendations on influenza vaccines for use in the Southern Hemisphere in 2016 and the Northern Hemisphere in 2016-2017.

PROTOCOL:

NOTE: The biosafety level (BSL) for the following protocol is BSL 2 for seasonal influenza viruses and BSL 3+ for potential pandemic influenza viruses. Viral titration is required in advance of a neutralization experiment to decide the viral concentration of the viral control (VC).

1. Virus titration

NOTE: Depending on the number of viruses and the number of duplicates, a well plate can be set up with the following flexibilities: (1) The viral dilution can be arranged along either the rows or the columns (Figure 1). Each virus should occupy a separate row/column. (2) The viral dilution increment is flexible, but it should start with the highest viral concentration at the top-left corner. (3) There is no restriction on the number of duplicates.

NOTE: Madin Darby canine kidney (MDCK) and MDCK-SIAT1 (SIAT) cells were kindly provided by Dr. M. Matrosovich, Marburg, Germany[10]. SIAT cells are MDCK cells stably transfected with the human CMP-N-acetylneuraminate: β-galactoside α-2,6-sialyltransferase gene for enhanced expression of sialic acid (SA) α2-6Gal-terminated oligosaccharides.

1.1 Aliquot sufficient MDCK cells or MDCK-SIAT cells[8] into 96-well plates (200 µL/well). Incubate the cells at 37 °C with 5% CO_2 for 2 or 3 days to reach confluence. Propagate the cells in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). Incubate the SIAT cells at 37 °C with 5% CO_2 and 1 mg/mL G418 sulphate.

NOTE: The dilution factor is dependent upon experience and the cell line used. The cell monolayer must be confluent (*i.e.,* no cell wall or visible outline for MDCK cells and a tightly-packed layout for MDCK-SIAT1 cells) at the time of virus inoculation. Examples of dilutions based on the subculture of confluent flasks of MDCK or MDCK-SIAT1 cells are given in Table 1.

1.2 Wash the cells 3 times with 200 µL of virus growth medium (VGM) per well. Sterilize the multiwall plate washer with 70% EtOH for 30 min, and then rinse it in sterile phosphate-buffered saline (PBS) or VGM before the wash.

1.3 Aspirate the VGM and immediately add 50 µL of VGM to each well.

1.4 Add 900 µL of VGM to each of 6 sterile tubes (or fewer, depending upon the number of test viruses and duplicates). Pipette 100 µL of virus into the first tube, mix well, and serially dilute, changing the tips between each dilution. Repeat for each virus to be titrated.

NOTE: This will give virus dilutions of 10^{-1} to 10^{-6}.

1.5 Add 50 µL of each virus dilution, starting at the highest dilution ($1x10^{-5}$ in Figure 1), to duplicated wells (Columns 9 and 10 in Figure 1) of a 96-well plate.

1.6 Place the plate at 37 °C for 2-3 h to allow the virus to infect the cells.
NOTE: A 2- to 3-h incubation is necessary to ensure that the viruses in the inoculum have enough time to reach the monolayer.

1.7 Prepare the overlay (10 mL/plate)
NOTE: The overlay consists of 5 mL of 2X DMEM, Trypsin (2µg/mL final concentration), 20 µL of 1 mg/mL stock, and 5 mL of cellulose cotton linters (see the materials and equipment table).

1.8 Remove the inoculum.

1.9 Add the overlay (200 µL to each well). Incubate overnight at 37 °C, UNDISTURBED.
NOTE: The virus budding takes place after approximately 4 h, so it is important that the plate is not disturbed after this time.

1.10 Aspirate the overlay from the wells.

1.11 Add ice-cold 4% paraformaldehyde in PBS A (200 µL/well). Place them at 4 °C for 30 min or at room temperature for 20 min.
NOTE: PBS A is natural-pH phosphate-buffered saline with 10 g of NaCl, 0.25 g of KCl, 1.437 g of Na_2HPO_4, 0.25 g of KH_2PO_4, and 1 L of distilled water (see the materials and equipment table).

NOTE: Paraformaldehyde is harmful when inhaled and can cause burns if swallowed. There is also limited evidence of a carcinogenic effect, and it may cause sensitization after skin contact.

1.12 Aspirate the paraformaldehyde and wash the plates twice with PBS A (200 µL/well).

1.13 Store the plates in PBS A at 4 °C for future use, or carry on with the following steps.

1.14 Add permeabilization buffer (100 µL/well). Leave it at room temperature for 30 min.

1.15 Wash plate twice with PBS A (200 µL/well).

1.16 Add 50 µL of the first antibody, mouse MAb against influenza type A (1:1000 in ELISA Buffer), per well. Incubate it at room temperature for 1 h (or 4 °C overnight), with shaking.

1.17 Wash it 3 times in 100 µL of wash buffer (0.05% Tween 80 in PBS A, v/v) per well. Incubate it at room temperature for 5 min between washes. Alternately, wash it 3 times without incubation using 300 µL per well .

1.18 Add 50 µL of the second antibody, goat anti-mouse IgG (H+L) HRP conjugate (1:1000 in ELISA Buffer), per well. Incubate it at room temperature for 1 h, with shaking.

1.19 Wash it 3 times as described in step 1.17.

1.20 Add the substrate (50 µL/well). Incubate it at room temperature for 30 min or until the development of a blue color is clearly visible.

1.21 To stop the reaction, wash the plate twice with 200 µL of distilled water per well, incubating at room temperature for 2 -3 min between the washes.

1.22 Air-dry the plate and store it in a dark place (*e.g.,* wrap it in aluminum foil).

2 Titration quantitation

2.1 Place a well plate in the scanning area of a flatbed scanner, as shown in Figure 2a. Use the L-shape position limit to ensure an optimum and repeatable imaging location.
NOTE: It is possible to image two well plates in one scan.

2.2 Scan an image.
NOTE: The settings are shown in Table 2.

2.3 Run the "Wellplate Reader" software to calculate the required virus concentration (Figure 3).

2.3.1 Click the "Load image" button to load an image. Slide the red bar in the histogram of the "Global Threshold" tab to adjust the sampling threshold. Click the "Update" button to examine the effect on the image.

2.3.2 Tick the "Calculate Neutralization/Titration" box. Click the "Sampling" button to quantify the ICP. Click the "Save" button to save the sampling results when prompted.
NOTE: After the sampling process, a new window called "Neutralization & Titration Calculation" appears automatically if the "Calculate Neutralization/Titration" box is ticked.

2.3.3 Load or input the well-plate definition map. Indicate the threshold (*e.g.,* 30%) in "Titration: Optimal Virus Population (%)". Select the "Titration Process" tab to calculate the titration results. Check the titration results. Click the "Save & Close" button to save the titration results.
NOTE: Refer to Supplementary S1 for more detailed instruction on the software. A copy of the bespoke software is available upon request.

NOTE: Typically, the best virus dilution for the plaque-reduction assay yields around 50% (20-85%) ICP of total cells within each well[11].

3. Virus neutralization

NOTE: Calculate the number of plates required for the neutralization assay. Each plate can accommodate one virus and a number of antisera.

NOTE: Depending on the number of antisera and the number of duplicates, a well plate can be set up with the following flexibilities: (1) The serum dilution can be arranged along either row or a column (Figure 4). Each serum should occupy a separate row or column. (2) The increment of serum dilution is flexible, but it should start with the highest serum concentration at the top-left corner of a plate. (3) The number of duplicates balances the number of antisera. More duplicates can help to smooth out experimental variations. (4) The number of the VC and the number of the cell control (CC) duplicates are flexible, but they should also be in separate rows or columns. It is expected that the number of VC duplicates is significantly greater than that of the antisera.

3.1 Prepare the cell monolayer, as in steps 1.1-1.3, 2 or 3 days in advance.

3.2 Add 50 µL of VGM to each well of the plate.

3.3 Use Columns 11 and 12 for VC and CC, respectively. Add 50-µL aliquots of 1:20 receptor-destroying enzyme (RDE)-treated serum to the first row (A) of Columns 1-10.
NOTE: For each virus and serum combination, the assay is performed in duplicate, so one plate may, for example, contain one virus tested against five antisera.

3.4 Perform 2-fold serial dilutions by transferring 50 µL from Row A to Row H (Columns 1-10 in Figure 4) and discarding 50 µL from Row H.

3.5 Add 50 µL of diluent to each well of the CC column (Column 12 in Figure 4).

3.6 Add 50 µL of the virus to each well of the plate, except for the CC column (Columns 1-11, Rows A-H on Figure 4).

3.7 Incubate it at 37 °C for 2-3 h. Remove the inoculum. Add the overlay (200 µL) to each well. Incubate it overnight at 37 °C, UNDISTURBED. Fix and stain the plates as for the virus titration (steps 1.11-1.22).

4 Neutralization quantification

4.1 Place a well plate in the scanning area of a flatbed scanner, as shown in Figure 2a. Use the L-shape position limit to ensure an optimum and repeatable imaging location.
NOTE: It is possible to image two well plates in one scan.

4.2 Scan the plate, as described in step 2.2.

4.3 Run the “Wellplate Reader” software to calculate the required viral titers (Figure 3, step 2.3).

4.3.1 Click the “Load image” button to load an image. Slide the red bar in “Global Threshold” to adjust the sampling threshold. Click the “Update” button to examine the effect.

4.3.2 Tick the “Calculate Neutralization/Titration” box. Click the “Sampling” button to quantify the ICP. Click the “Save” button to save the sampling results when prompted.
NOTE: After the sampling process, a new window called “Neutralization & Titration Calculation” appears automatically if the “Calculate Neutralization/Titration” box is ticked.

4.3.3 Load or input the well-plate map. Indicate the threshold (*e.g.,* 50%) in “Neutralization: Infection Deduction (%).”

4.3.4 Select “Neutralization Process” to calculate the titers. Check the titers and click the “Save & Close” button to save the neutralization results.
NOTE: Neutralization is reported as the reciprocal of the highest dilution of the serum with the predefined ICP reduction (such as 50% or 80%) against the VC (the mean of Column 11 in Figure 4). A 50% ICP reduction was used in the Representative Results.

REPRESENTATIVE RESULTS:
Following the above procedures, we present some results from recent antigenic characterization experiments. The titration setup was designed to examine eight input viruses, with double duplicates for each dilution. The viral dilutions were tested between $1.0x10^{-1}$ and $1.0x1.0^{-6}$ to cover the variations between the viruses. The test viruses were from the H3N2 subtype, including A/Cameroon/15V-3538/2015, A/Vladivostok/36/2015, A/Moscow/103/2015, A/Tomsk/5/2015/, A/Moscow/101/2015, A/Moscow/100/2015, A/Moscow/133/2015, and A/Bratislava/437/2015. The titration results are illustrated in Figure 5. Figure 5d demonstrates the decrease of ICPs with the increase of virus dilution. The curves were normalized against the ICPs of the same viruses that yielded infections in all cells within a well[12] (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).

Neutralization aimed to have one input virus against five antisera, with double duplicates on each plate. The reference virus shown is H3N2 A/Stockholm/63/2015. The five antisera are A/HK 4801/14 Egg F12/15, A/HK 7295/14 MDCK F02/15, A/South Africa R2665/15 SIAT F50/15, A/Swiss 9715923/13 SIAT NIBF F18/15, and A/Dutch 525/14 SIAT f23/15. The neutralization results are illustrated in Figure 6. Figure 6d shows the infection progress with the increase of serum dilution. The normalized positive population on the vertical axis represents the ratio of ICPs from the corresponding antisera response against the average ICP of the reference virus[12]. The background ICP from uninfected cell controls was subtracted during the normalization. The neutralization titers were determined as the reciprocals of the antiserum dilutions corresponding to 50% ICP reduction (Table 4). Linear interpolation was used to estimate titers falling between two adjacent serum dilutions.

FIGURE LEGENDS:
Figure 1. Example of a well-plate setup in a virus titration experiment. Test viruses are assigned to separate rows (A to H). Columns are designed for different viral dilutions (1 to 12). Two columns were used as duplicates for each viral dilution. Scale bar: 10 mm.

Figure 2. Schematics of a sample scanning system. (a) A 96-well plate on the imaging position of a flatbed scanner (republished from Reference 11 with permission from Elsevier B.V.), and (b) dimensions of the L-shape position limit shown in (a).

Figure 3. Desktop diagram of the "Wellplate Reader" quantitation software.

Figure 4. Example of a well-plate setup in a neutralization experiment. Each antiserum is assigned to two columns with duplicates (1 to 10). Rows are designed for different serum dilutions (A to H). The virus control takes Column 11, with eight duplicates (A11 to H11). The cell control is in Column 12, with eight duplicates (A12 to H12). Scale bar: 10 mm.

Figure 5. Results of a titration experiment. (a) The well-plate setup, (b) scanned well-plate image, (c) illustration of the color-encoded, quantitated, virus-infected cell population, and (d) normalized virus-infected cell populations against virus dilutions. 30% ICP was used as the threshold. The error bars in (d) are standard deviations (SD) from the sample duplications (± 1 SD). Scale bars in (b) and (c): 10 mm.

Figure 6. Results of a neutralization experiment. (a) The well-plate setup, (b) scanned well-plate image, (c) illustration of the quantitated, virus-infected cell population, and (d) normalized virus-infected cell population against the serum dilution. The input virus (VC) is A/Stockholm/63/2015. 50% ICP was used to calculate the titers. The error bars in (d) are standard deviations (SD) from the sample duplications (± 1 SD). Scale bars in (b) and (c): 10 mm.

TABLE LEGENDS:
Table 1. Typical dilutions on a subculture of confluent flasks of MDCK or MDCK-SIAT1 cells.

Table 2. Typical setup of a flatbed scanner.

Table 3. Viral dilutions calculated from a population of 30% ICP.

Table 4. Titers at 50% ICP of A/Stockholm/63/2015 against antisera.

DISCUSSION:
In this study, we described an imaging-based MN assay to quantify the true antigenic relationships between viruses. Compared with other plaque-reduction assays, the method emphasizes measuring the ICP of an entire well, ensuring the complete coverage of the infective population. Its independence of plaque formation also widens the application of the assay to viruses that can form mainly invisible small plaques or that can only manage single-cell infection. Therefore, the assay is capable of examining more viruses and the effects of a wider range of antibodies than HI, and it helps to more comprehensively reflect the antigenic similarities or differences between viruses[12]. For example, when oseltamivir carboxylate is included, the MN assay is less affected by NA-dependent binding, reflecting the antigenic differences more accurately. During the development of the assay, great efforts were made to enhance the experiment consistency, speed, and detection

sensitivity, including by controlling input viruses through quantitated titration, imaging in high throughput with a flatbed scanner, and implementing a user-friendly data processing platform. Results have generally been consistent with and confirmed those of HI. Notably, the results also revealed antigenic differences between antigenic drift variants of recent type A and B vaccine viruses, as well as antigenic changes caused by culture-selected or sporadic changes, such as the G155E substitution in HA1 of certain A(H1N1)pdm09 viruses[12].

The protocol matched the virus type or subtype with the selection of cell lines that gave the strongest infection, which greatly enhanced the imaging signal. Experimental variation was smoothed with the design of duplicates that balanced with the number of tested antisera. Uncertainties can also come from the image quality, which is mainly influenced by the imaging device. A decent color flatbed scanner can significantly improve the image contrast and reduce the noise. The amount of input virus in the neutralization must be quantitatively determined in advance from the corresponding titration while the viruses still maintain a similar status. During neutralization, the antiserum response to an infection is normalized against the difference between the reference virus (VC) and the background level (CC). Accurate measurements of VC and CC are essential to a neutralization experiment. More duplicates are recommended (eight duplicates were used in the Representative Results). Finally, understanding the software is vital to the success of an experiment. The software has been tested for routine virus characterization in the WHO Influenza Centre, UK for more than two years. Detailed instructions for dealing with various situations is provided in the Supplementary S1.

This MN assay is suitable for applications where the samples cover an extremely large field of view but only require moderate imaging resolution. The low cost and simple setup make the system readily available to most virology laboratories. The variation in measurements of the same sample was less than 3%, which roughly defines the uncertainty introduced during the scanning[11]. Such reproducibility is sufficient in many virological studies and can be further reduced by averaging images from multiple scans.

In conclusion, this study demonstrates a robust MN assay that can be routinely used in antigenic study and to support HI data in detailed analyses of currently-circulating influenza viruses, notably for the biannual selection of viruses for inclusion in human influenza vaccines.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure

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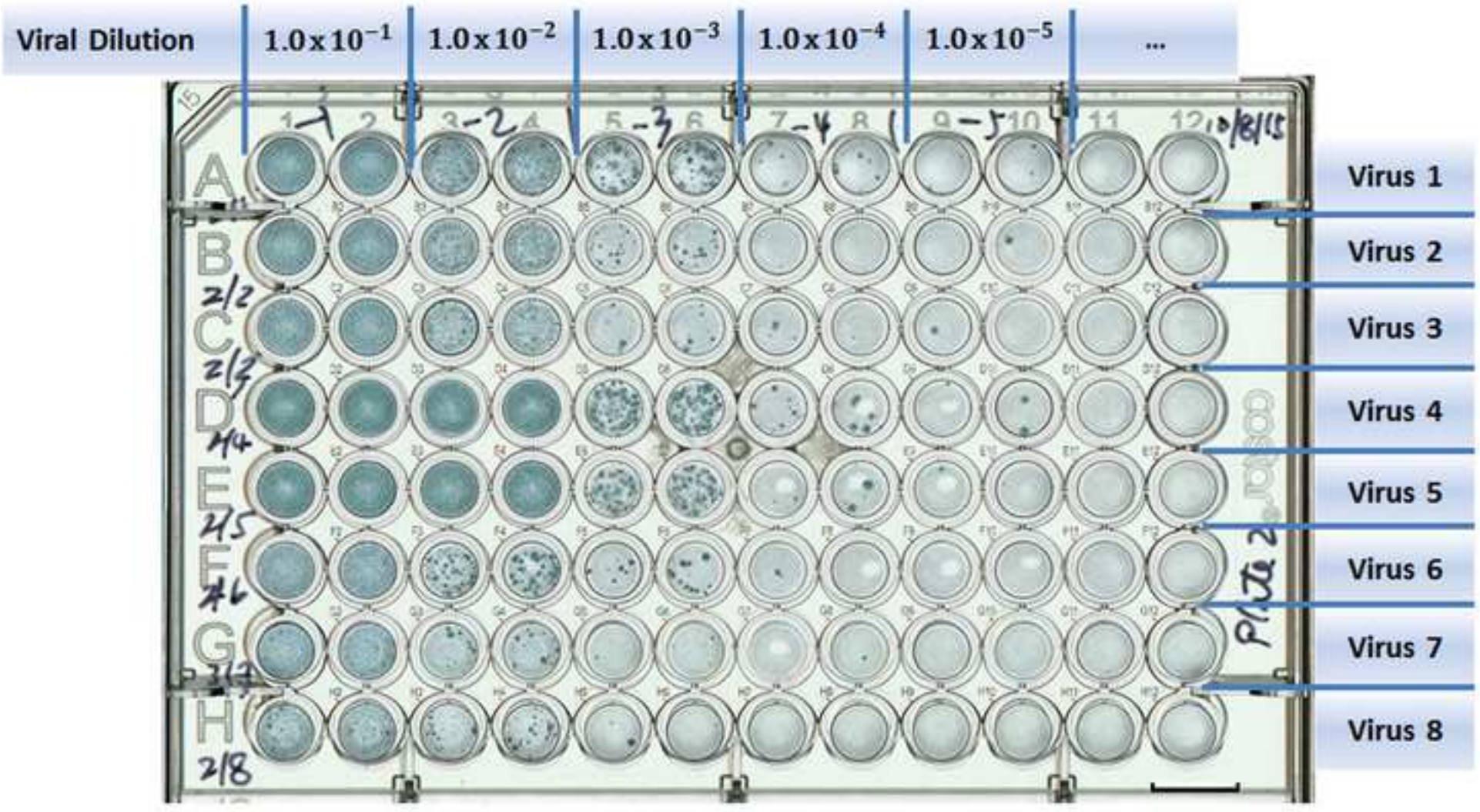

Figure

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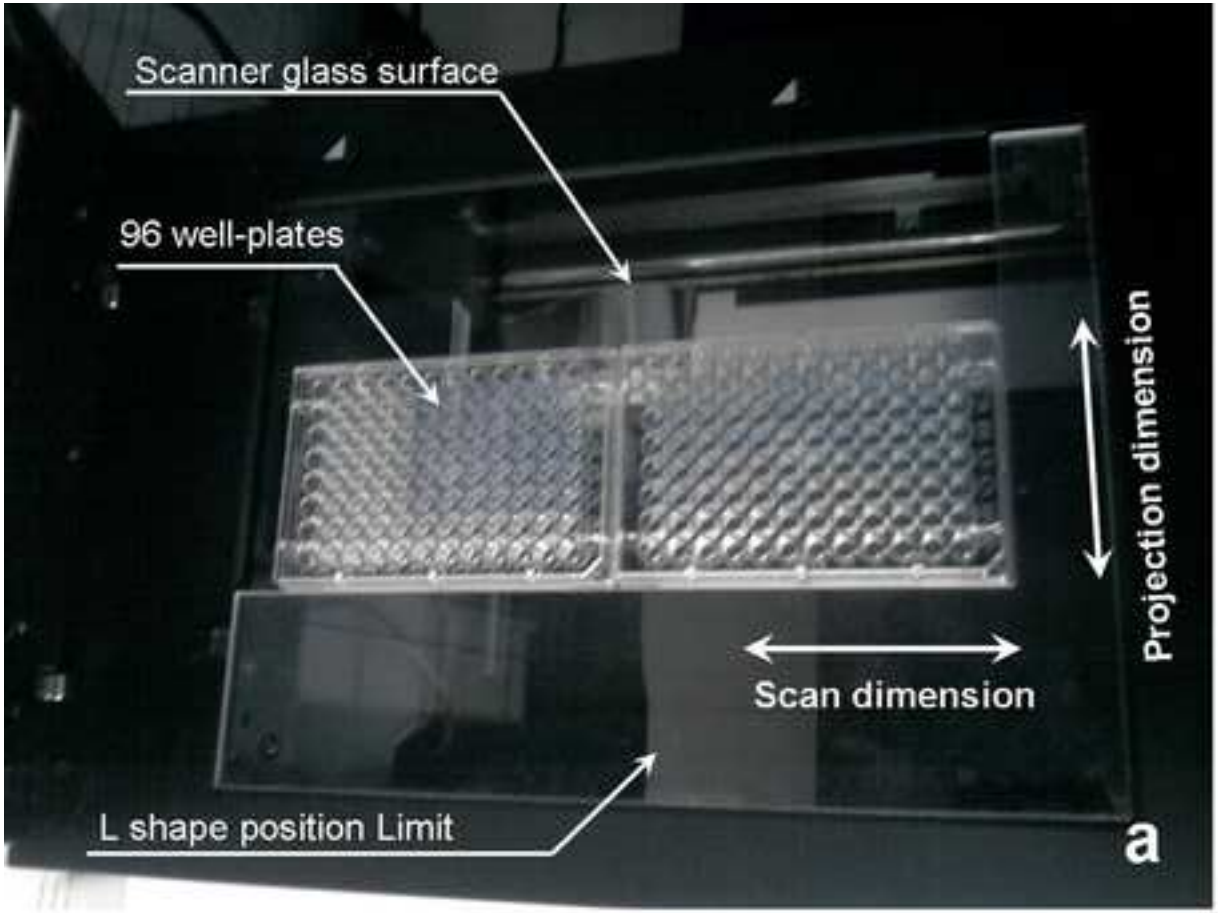

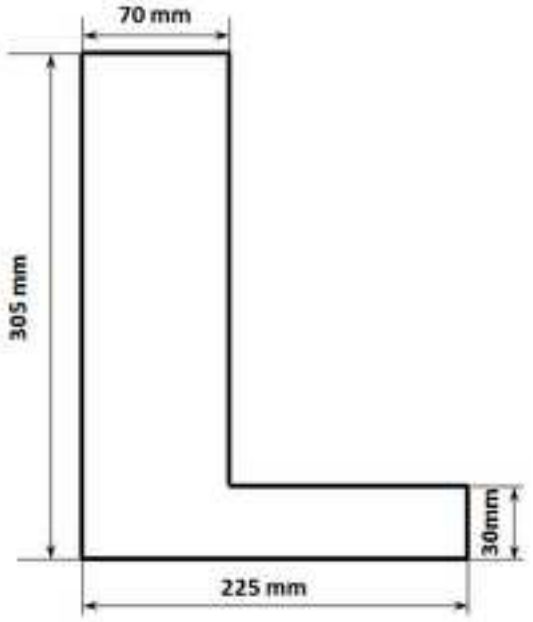

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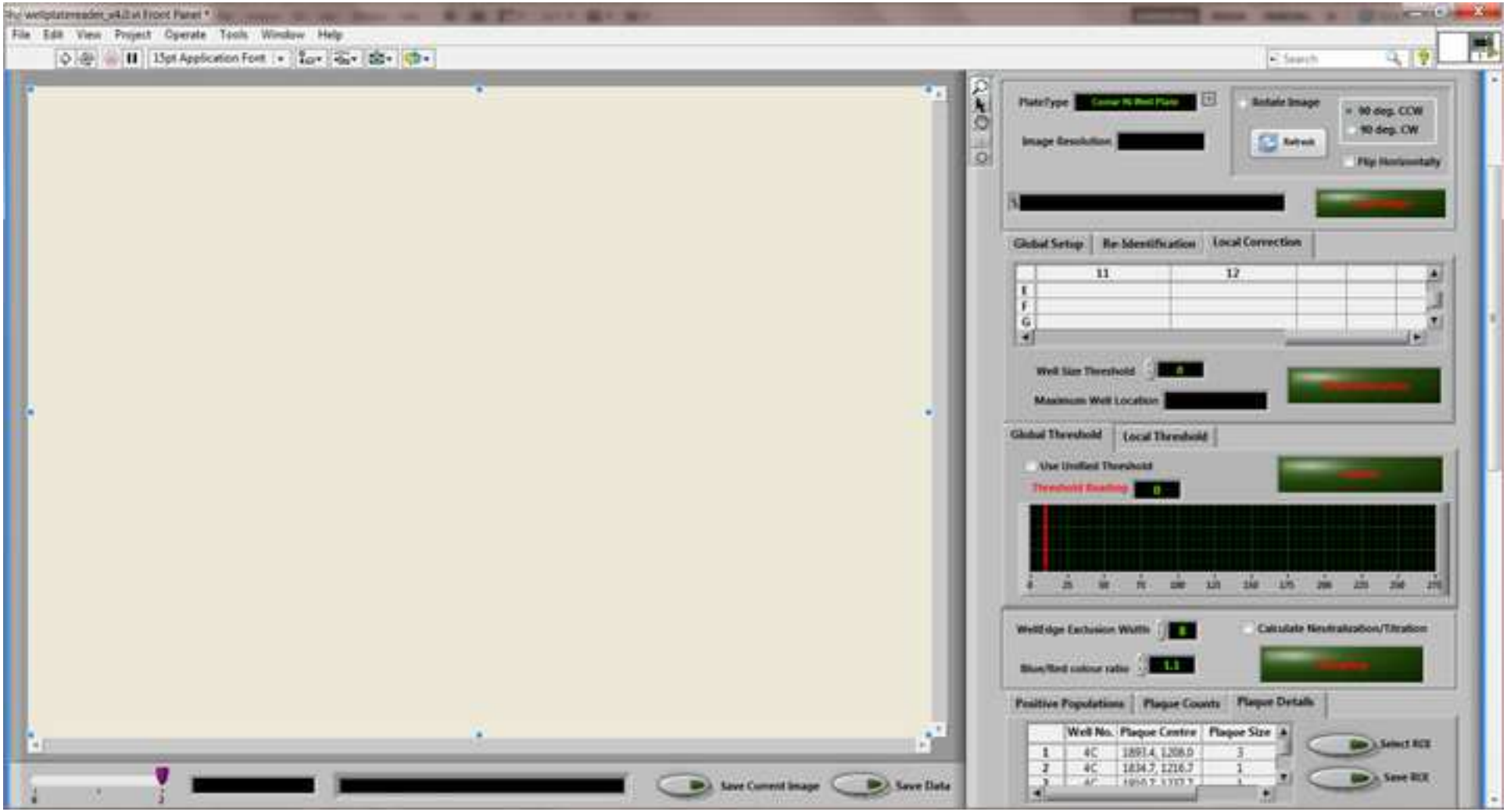

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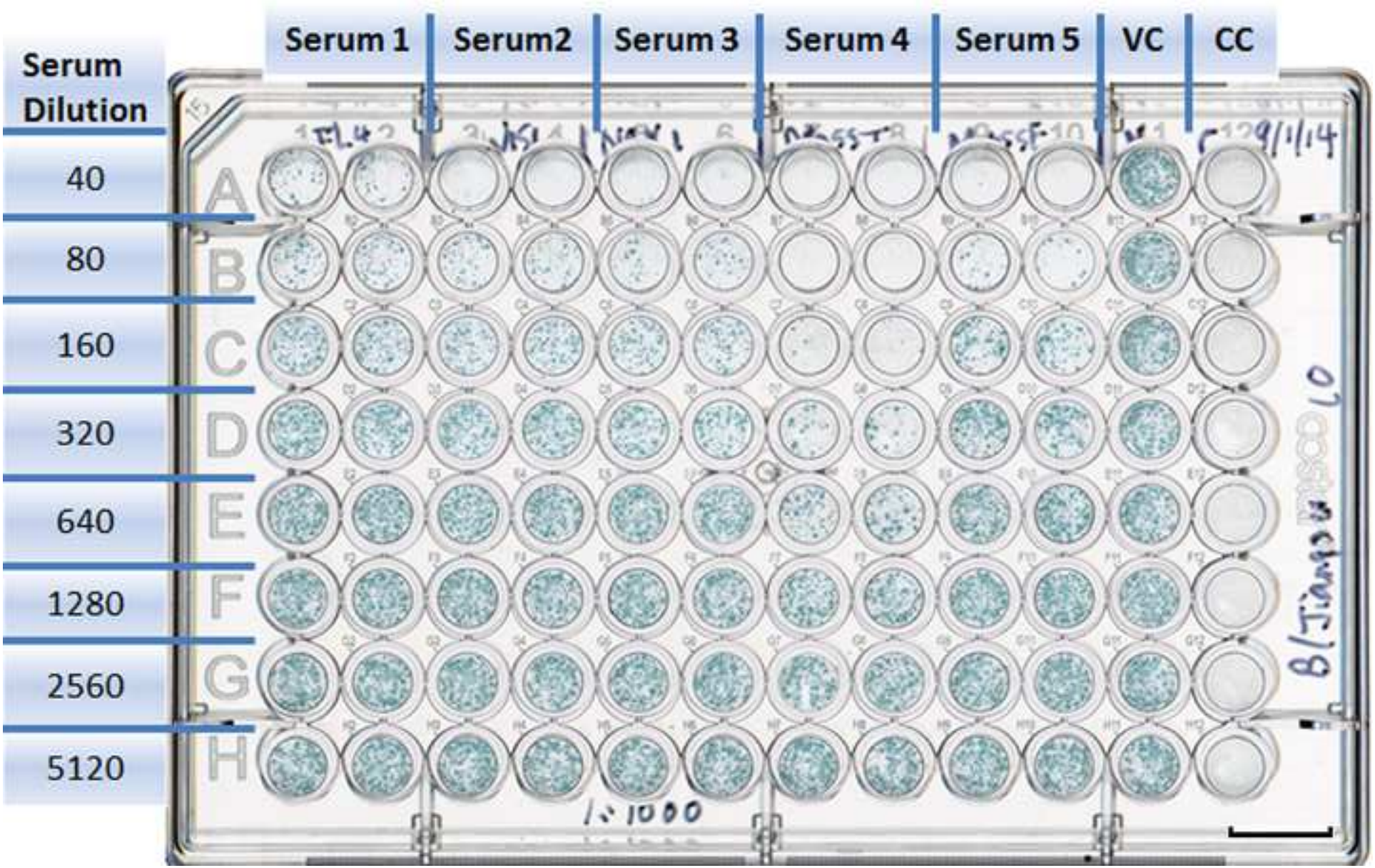

Figure

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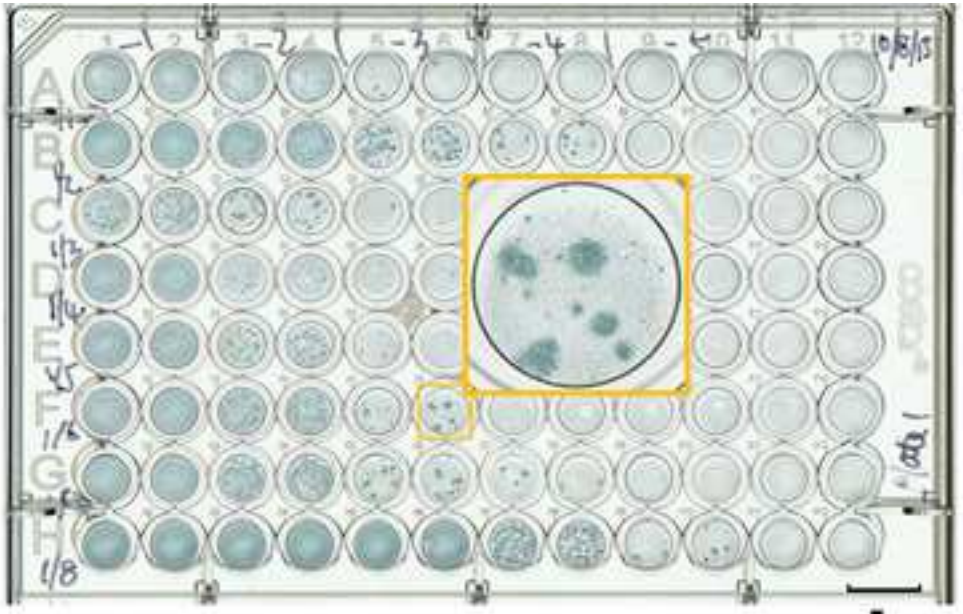
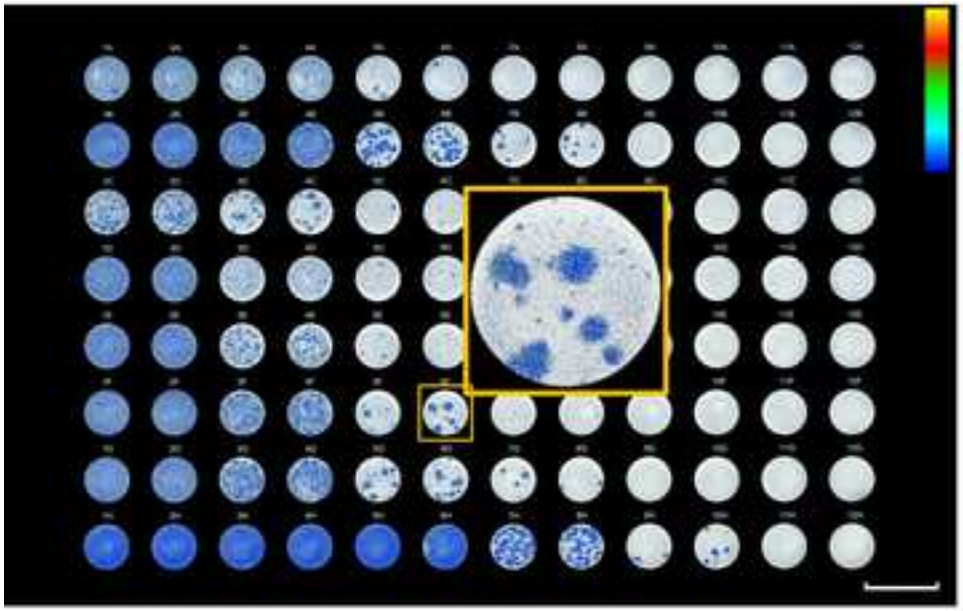
Titration plate setup

	1	2	3	4	5	6	7	8	9	10	11	12
Virus Dilution	1.0X10^{-1}		1.0X10^{-2}		1.0X10^{-3}		1.0X10^{-4}		1.0X10^{-5}		1.0X10^{-6}	
A	A/Cameroon/15V-3538/2015											
B	A/Vladivostok/36/2015											
C	A/Moscow/103/2015											
D	A/Tomsk/5/2015											
E	A/Moscow/101/2015											
F	A/Moscow/100/2015											
G	A/Moscow/133/2015											
H	A/Bratislava/437/2015											

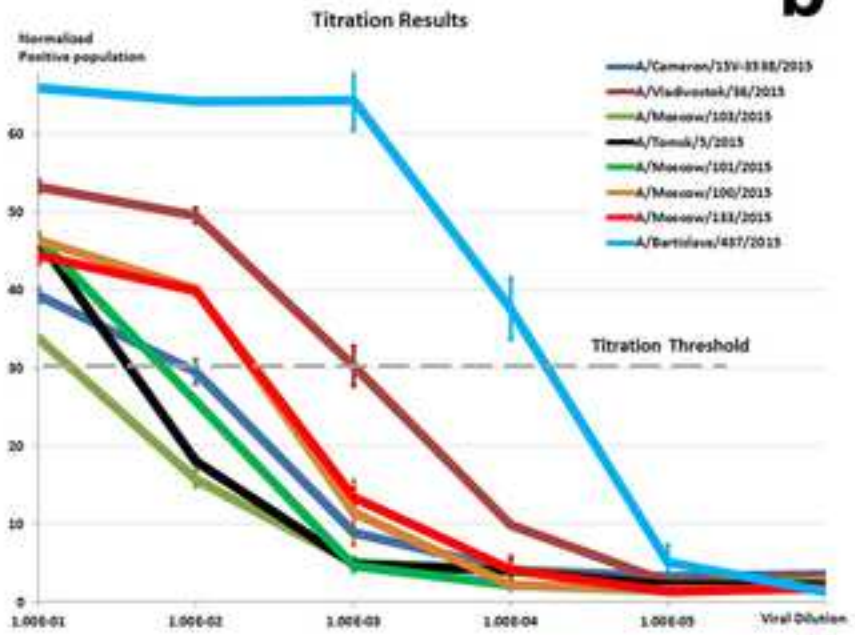
a



b



c



d

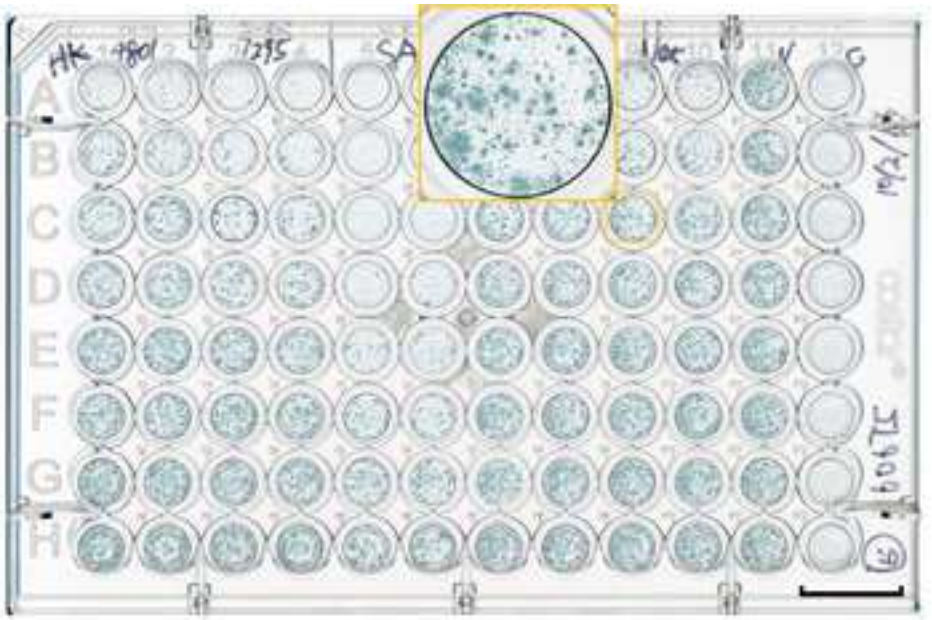
Figure

Click here to download Figure figure6.jpg

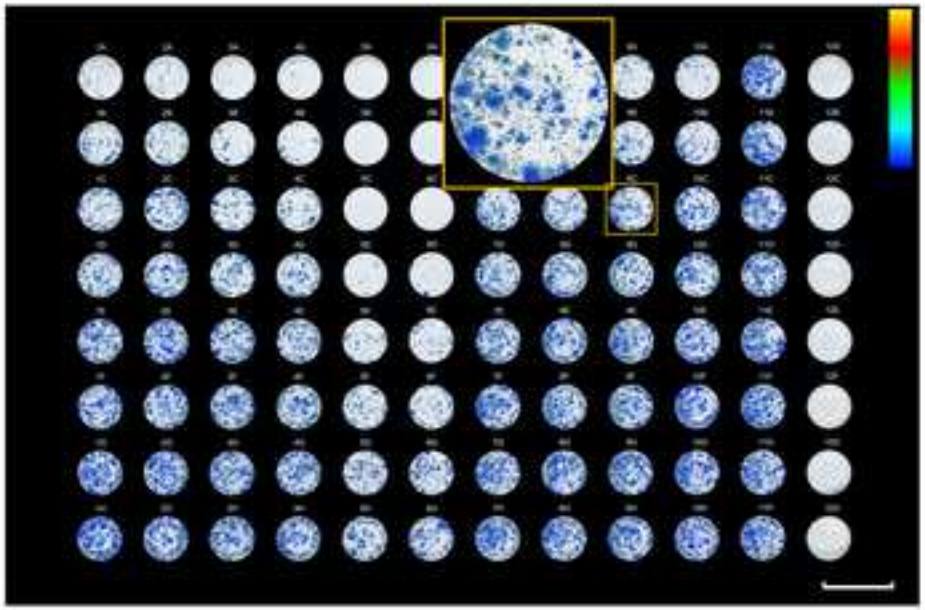
Neutrolisation plate setup

Serum / Dilution	1	2	3	4	5	6	7	8	9	10	11	12
A 40	Serum 1 A/HK 4801/14 Egg F12/15		Serum 2 A/HK 7295/14 MDCK F02/15		Serum 3 A/South Africa R2665/15 SIAT F50/15		Serum 4 A/Swiss 9715923/13 SIAT NIB F18/15		Serum 5 A/Dutch 525/14 SIAT F23/15		Virus Control	Cell Control
B 80												
C 160												
D 320												
E 640												
F 1280												
G 2560												
H 5120												

a



b



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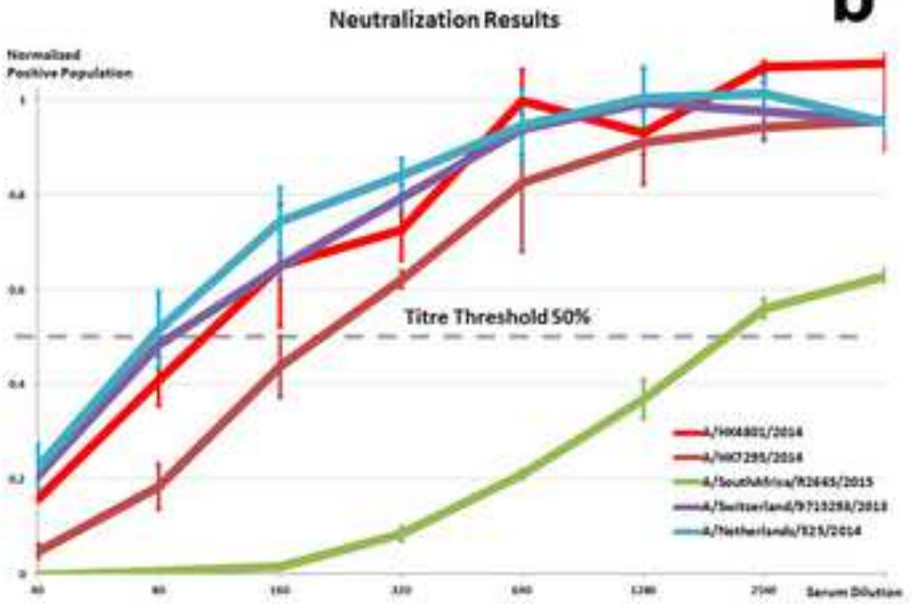

d

Table

Typical dilution		
	MDCK cells	**MDCK-SIAT1 cells**
2 days	1:5 (1 + 4)	1:10 (1 + 9)
3 days	1:10 (1 + 9)	1:20 (1 + 19)
Typical number of cells per ml		
	MDCK cells	**MDCK-SIAT1 cells**
2 days	2×10^5	1×10^5
3 days	1×10^5	5×10^4

Table

Click here to download Table Table2.xlsx

Mode	Professional Mode
Document Type	Film (with Film Area Guide)
Auto Exposure Type	Photo
Image Type	24-bit Color
Resolution	1200 dpi
Saved image Format	TIFF

Table

Row	Virus	Recommended dilution
A	A/Cameron/15V-3538/2016	1.0×10^{-2}
B	A/Vladivostok/36/2015	1.0×10^{-3}
C	A/Moscow/103/2015	1.1×10^{-1}
D	A/Tomsk/5/2015	5.9×10^{-1}
E	A/Moscow/101/2015	8.3×10^{-1}
F	A/Moscow/100/2015	1.4×10^{-2}
G	A/Moscow/133/2015	1.3×10^{-2}
H	A/Bratislava/437/2015	1.3×10^{-4}

Table

Column	Antisera	Recommended titre
1 - 2	A/HK4801/2014	110
3 - 4	A/HK7295/2014	213.3
5 - 6	A/SouthAfrica/R2665/2015	2155.8
7 - 8	A/Switzerland/9715293/2013	89.4
9 - 10	A/Netherlands/525/2014	78.6

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment Material and Equipment Table.xlsx

Name	Company
VGM	Sigma
PBS A	
Avicell	FMC
2XDMEM	Gibco
Trypsin	Sigma
Overlay (10ml/plate):	
Triton X- 100^{e}	Sigma
Tween 80	Sigma
Horse serum	PAA Labs Ltd
Mouse MAb against influenza type A	Biorad
Goat anti-mouse IgG (H+L) HRP conjugate	Biorad
True Blu peroxidase substrate	KPL
96-well flat-bottom microtitre plates	Costar
8 channel multiwall-plate washer and manifold	Sigma
Perfection Plate Scanner	Epson

Software operational environment: LabVIEW	National Instruments Corporation

Catalogue Number
D6429, P0781
RC-581F
21935-028
T1426
T8787
P5188
B15-021
MCA 400
172-1011
50-78-02
3596
M2656
V750 Pro

Window based with NI Vision builder

Comments
500 ml DMEM+5 ml Pen/Strep[b]
Nature pH Phosphate-buffered saline: NaCl -10 gm, KCl - 0.25 gm, Na_2HPO_4 - 1.437 gm, KH_2PO_4 – 0.25 .gm, and Dist. Water – 1 L.
2.4g in 100ml distilled water dissolved by agitation on a magnetic stirrer for 1 hr. Sterilize by autoclaving.
5ml 2X DMEM, Trypsin 2µg/ml final conccentration,Avicell - 5ml
Permeabilisation buffer: 0.2% in PBS A (v/v)
Wash Buffer: 0.05% Tween – 80 in PBS A (v/v)
ELISA Buffer: 10% in PBS A (v/v) + 0.1% Tween 80
1st Antibody: 1:1000 in ELISA Buffer
2nd Antibody: 1:1000 in ELISA Buffer
Substrate: True blue +0.03% H_2O_2[g] (1:1000 of 30% solution)
The imaging software can be downloaded freely from http://www.epson.com/cgi-bin/Store/support/supDetail.jsp?oid=66134&infoType=Downloads.

Version: LabVIEW2012 or above

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Author(s): Lin, Yipu, Gu, Yan, McCauley, John

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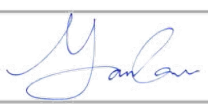
Signature:	
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v1.8

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Response to comments

Please find response after each corresponding comment in color.

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, Na_2HPO_4 - 1.437 gm, KH_2PO_4 – 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 well[12] (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 virus[12]. 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.

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  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 viruses[12].

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