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TITLE:

An Improved and High Throughput Respiratory Syncytial Virus (RSV) Micro-neutralization Assay

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KEYWORDS:

Respiratory syncytial virus, neutralizing antibody, antibody, plaque reduction, infectious disease, standardization

SUMMARY:

This study describes a high throughput, imaging-based micro-neutralization assay to determine the titer of neutralizing antibodies specific for respiratory syncytial virus (RSV). This assay format has been tested on different sample types.

ABSTRACT:

Respiratory syncytial virus-specific neutralizing antibodies (RSV NAbs) are an important marker of protection against RSV. A number of different assay formats are currently in use worldwide so there is a need for an accurate and high-throughput method for measuring RSV NAbs. We describe here an imaging-based micro-neutralization assay that has been tested on RSV subgroup A and can also be adapted for RSV subgroup B and different sample types. This method is highly reproducible, with inter-assay variations for the reference antiserum being less than 10%. We believe this assay can be readily established in many laboratories worldwide at relatively low cost. Development of an improved, high-throughput assay that measures RSV NAbs represents a

significant step forward for the standardization of this method internationally as well as being critical for the evaluation of novel RSV vaccine candidates in the future.

INTRODUCTION:

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infections in the pediatric population worldwide[1]. Despite its high burden, there is still no vaccine or treatment available. Since 2013, the World Health Organization (WHO) has declared RSV vaccine development as a major research priority, with annual WHO consultation meetings[2,3]. The WHO has agreed on using RSV neutralizing antibody (NAb) measurement to monitor vaccine immunogenicity, as this is recognized as the major serological marker of protection[4]. NAbs have been shown to protect against severe RSV infection in a number of studies as well as clinical trials of the anti-RSV monoclonal antibody palivizumab, currently the only prophylactic strategy available[4].

There are multiple NAb assay formats used by laboratories worldwide, including cell-based and molecular-based assays, which have made standardization efforts challenging[5-8]. However, the conventional plaque-reduction neutralization (PRN) assay that measures the number of reduced plaque forming units (PFU) by the presence of an RSV-specific antibody still remains the gold standard[9]. Here, we report an improved, simplified, and high-throughput PRN protocol that can be used on numerous cell lines, for different RSV strains and with increased assay throughput. This protocol has been tested using clinical samples from different settings as well as on samples from animal model experiments.

PROTOCOL:

NOTE: All steps have to be performed in a BSL2 hood unless stated differently. Viral titration is required in advance of a PRN assay to determine the optimal RSV concentration used in the PRN assay. It is recommended to aliquot the virus stocks in a small volume that will be thawed once and used for each NAb assay. Using the same viral stock for all NAb assays performed for all samples from one study is also recommended. Make sure culture media and phosphate-buffered saline (PBS) is warmed at 37 °C before adding to cell plates.

1. RSV Viral Titration

NOTE: Depending on the number of virus stocks and the number of duplicates, the assay plate can be set up according to **Figure 1**. Each virus stock should be titrated in triplicate down the assay plate, starting at the highest viral concentration (*i.e.*, 1:10). Serial titrations can be typically 1:10. A549 cell culture and maintenance as well as RSV culture procedure are done using standard procedures and are not included in this protocol.

1.1 Seeding plates (Day 1)

1.1.1 Resuspend A549 cells in Dulbecco's Modified Eagles medium (DMEM) + 10% fetal calf serum (FCS) + 1000 IU penicillin/streptomycin (pen/strep) at 4 x 10^5/mL. Seed 96-well flat-bottom sterile plates with 100 µL/well containing 4 x 10^4 A459 cells.

1.1.2 Incubate plates overnight at 37 °C, 5% CO_2 (cells will be in the log-phase growth).

NOTE: Use new A549 cell vial after 23 passages. It is recommended to not start the experiments when a new cell vial is still at the first three passages.

1.2. Virus infection (Day 2)

1.2.1 Preparation of virus serial dilution

1.2.1.1 Rapidly thaw a single frozen vial of RSV in a 37 °C water bath until almost completely thawed and place immediately on ice. Prepare 3 replicates for each RSV virus stock to be assayed, use an initial virus stock dilution of 1:10 with 100 µL of diluted virus/well and reserve at least one column for negative control.

NOTE: **Figure 1** shows negative control in triplicates.

1.2.1.2 Add 100 µL of each diluted virus at 1:10 in triplicate of row A of a 96-well U-bottom sterile plate. Add 90 µL/well of DMEM + 1000 IU pen/strep without FCS to rows B to H.

1.2.1.3 Perform serial 1:10 dilutions down the plate by transferring 10 µL from row A to row B. Mix solution by pipetting content up and down 5 times and continue the ten-fold dilutions until row H. Discard the final 10 µL so that the final volume in each well is 90 µL.

NOTE: It is important to use new tips for each dilution.

1.2.2 Virus inoculation to A549 cells

1.2.2.1 Retrieve A549 cell plate(s) prepared on the previous day. Ensure that A549 cell monolayers in the 96-well plates are ~80% confluent. Discard media by gently inverting plate and lightly blotting on sterile absorbent paper towel.

1.2.2.2 Wash all wells with 100 µL of PBS twice, discard excess PBS by gently inverting plate and lightly blotting on sterile absorbent paper towel after each wash. Do not allow plates to dry.

1.2.2.3 Transfer the virus dilutions from the viral titration plate (**Figure 1**) to corresponding wells on the A549 cell plate. Incubate the plate for 1 h at 37 °C, 5% CO_2. After 1 h incubation, decant supernatants using a pipette (to avoid cross contamination). Take out 90 µL/well.

1.2.2.4 Add 100 µL of 1x medium 199 (M199, see **Table of Materials**) + 1.5% carboxymethylcellulose sodium salt (CMC) low viscosity + 2% FCS containing pen/strep to each well.

NOTE: 2x M199 + 4% FCS + 2% pen/strep and 3% CMC solutions need to be prepared in advance and stored at 4 °C for future use. Make sure the solution is warmed at 37 °C before adding to plates.

1.2.2.4.1 Prepare 2x M199 solution: 1 sachet/500 mL distilled water + 20 mL FCS + 10 mL pen/strep (to make up 2x MI99). Filter the solution using a 0.22 µm filter unit.

1.2.2.4.2 For preparation of 3% CMC, add 15 g of CMC slowly into 500 mL of distilled water. Dissolve the CMC in water by using metallic stirrer heater at 50 °C, which takes about 1 h of preparation. Mix well to make 3% CMC and autoclave the solution.

NOTE: The CMC solid must be added to the water in order to be dissolved; adding water to the dry solid produces a "clump" of solid that is very difficult to dissolve.

1.2.2.4.3 Prepare 1x M199 + 1.5% CMC low viscosity + 2% FCS containing pen/strep by mixing 1:1 the 2x M199 and 3% CMC prepared in step 1.2.2.4.2.

1.2.2.5 Incubate plate for 3 days at 37 °C, 5% CO_2.

1.3 Developing assay plate and analysis (Day 5)

1.3.1 Fixation and developing assay plate

1.3.1.1 Discard M199 + CMC + 2% FCS containing pen/strep by gently inverting the plate. Add slowly (in order to avoid disturb the cell layer) 200 µL of fixation buffer (80% acetone, 20% PBS, stored at -20 °C) to fix cells. Incubate at -20 °C for 20 min.

1.3.1.2 Discard the fixation buffer and gently blot on absorbent paper towel. Leave plate face down to dry for 10 min.

NOTE: From this step onwards, assay can be performed outside the BSL2 hood.

1.3.1.3 Prepare 5% milk diluent blocking solution in filtered PBS containing 0.05% polysorbate 20 (PBS-polysorbate, see **Table of Materials**). For one plate, calculate the volume needed for blocking based on 200 µL/well and 110 wells: 200 µL/well x 110 wells = 22 mL (1.1 mL concentrated milk diluent in 20.9 mL filtered PBS-polysorbate). At this stage, also make up sufficient blocking solution for primary and secondary antibodies. For one plate, calculate the volume needed for each antibody solution based on 50 µL/well and 110 wells: 50 µL/well x 110 wells = 5.5 mL. Therefore, the total volume of milk diluent blocking solution required for a single plate assay is 33 mL.

1.3.1.4 Add 200 µL/well of blocking solution. Incubate plate at room temperature (RT) for 30 min. Discard the solution by gently inverting the plate and blot on absorbent paper towel.

1.3.1.5 Prepare 1:500 Goat X RSV antibody (mAb – primary antibody) diluted in blocking solution. Add 50 µL/well of the mAb solution and incubate at 37 °C for 1 h. Wash plate 3 times with filtered PBS-polysorbate.

1.3.1.6 Prepare 1:5,000 Alexa-Fluor donkey anti-goat IgG (secondary antibody) diluted in blocking solution. Add 50 µL/well of the secondary antibody solution and incubate at 37 °C for 1 h. Wash plate 5 times with filtered PBS-polysorbate.

1.3.1.7 Read plate on an automated spots reader using the fluorescein isothiocyanate (FITC) channel and count settings as shown in **Figure 2**. Wrap plate in foil and store at 4 °C. Calibrate the instrument using an unused 96-well culture plate and inputting count settings before assay.

NOTE: Re-scanning within a few days is possible if required. The calibration and count setting can be saved and used for the future scanning if the assay uses the same type of plate used for the calibration.

1.3.1.8 Check the images of wells for artefact plaques or disrupted cell monolayers (**Figure 3**). Exclude wells with disrupted cell monolayers.

NOTE: Depending on the size of the artefact plaques, manual counts may need to be performed for those wells or those wells may need to be discarded from data analysis. Criteria for a valid result include no disrupted cell monolayer or artefact plaques detected in more than one replicate well and no PFU detected in negative wells (wells without added virus).

1.3.2 Determine viral concentrations

1.3.2.1 Choose the last two dilutions where spots can be counted clearly. Calculate the average number of spots from replicate wells at the same dilution. Calculate the viral titer (PFU per mL) of the stock sample using the formula below:

PFU/mL = Average number of plaques/(Dilution factor × Volume of diluted virus added to the well)

NOTE: The virus concentration determined for each RSV stock can now be used in the PRN assay (step 2).

2. RSV Neutralization Assay

2.1 Seeding plates (Day 1)

2.1.1 Resuspend A549 cells in DMEM + 10% FCS + 1000 IU pen/strep at 4 x 10^5/mL. Seed 96-well flat-bottom sterile plates with 100 µL/well containing 4 x 10^4 A459 cells and incubate plates overnight at 37 °C, 5% CO_2 (cells will be in the log-phase growth).

NOTE: Use new A549 cell vial after 23 passages. It is not recommended to use A549 cells before passage number 3.

2.2 Virus and serum preparation (Day 2)

NOTE: Depending on the sample type, there are required steps for pre-processing samples before the NAb assay. It is recommended to use the RSV international standard sera that are now available upon request from the National Institute for Biological Standards and Controls (NIBSC).

2.2.1 Preparation of serum dilution

2.2.1.1 Thaw the human RSV reference antiserum (reference serum, REF) and serum test samples at RT. Heat-inactivate serum samples and reference antiserum in a water bath at 56 °C for 30 min prior to use. Prepare dilutions as per the plate template (**Figure 4**).

2.2.1.2 Prepare 1:100 dilution of the REF. Prepare a minimum of 110 µL of REF diluted in DMEM + pen/strep without FCS per assay plate (*e.g.,* 1.5 µL of REF in a total volume of 150 µL). Add 110 µL of 1:100 diluted REF in well A12 of a 96-well U-bottom sterile plate.

2.2.1.3 Add 55 µL of DMEM + pen/strep without FCS to wells B12 to H12 in column 12. Perform serial 1:2 dilutions down the plate by transferring 55 µL from row A to row B, mixing solution by pipetting content up and down 5 times and continuing until row H. Discard the final 55 µL of media so that the final volume in each well is 55 µL. Use new tips for each dilution.

2.2.1.4 Prepare 1:100 dilution of serum test samples. As all serum samples are assayed in triplicate, prepare a minimum volume of 110 µL/well x 3 = 330 µL per serum test sample.

2.2.1.5 Add 110 µL of each diluted serum test sample (1:100; S1=serum 1, S2= serum 2, *etc*.) to corresponding wells A1 to A9 and also E1 to E9 of the 96-well sterile plate according to **Figure 4**.

2.2.1.6 Add 55 µL of DMEM + pen/strep without FCS to all wells labelled X. Perform serial 1:2 dilutions as per step 2.2.1.3 until row D (for samples 1, 2, and 3). Discard the final 55 µL of media so that the final volume in each well is 55 µL. Similarly, perform the serial 1:2 dilutions for samples 4, 5, and 6 for rows E to H.

NOTE: It is important to use new tips for each dilution.

2.2.1.7 Add 55 µL of DMEM + pen/strep without FCS to wells in columns 10 and 11.

2.2.2 Preparation of virus

2.2.2.1 Rapidly thaw a single frozen vial of RSV in a 37 °C water bath until almost completely thawed and place immediately on ice.

2.2.2.2 Dilute virus in DMEM + pen/strep without FCS based on the concentration of the RSV aliquot determined in step 1.3.2. Apply a dilution that gives approximately 200 PFU/well. Prepare a total volume of 5.5 mL (for 100 wells).

2.2.3 Preparation of virus-serum mixture

2.2.3.1 Add 55 µL of the diluted virus to all wells of columns 1-9 and wells of rows E-H of columns 10 and 11 (positive control) according to the plate template (**Figure 4**).

2.2.3.2 Add 55 µL of DMEM + pen/strep without FCS to all wells of rows A-D of columns 10 and 11 (negative control). Incubate the plate for 1 h at 37 °C, 5% CO_2.

2.2.4 Virus inoculation to A549 cells

2.2.4.1 Prior to the completion of incubation period, retrieve A549 cell plate(s) prepared in step 2.1.1. Ensure that A549 cell monolayers in the 96-well plates are ~80% confluent.

2.2.4.2 Discard media by gently inverting plate and lightly blotting on sterile absorbent paper towel. Wash all wells with 100 µL of PBS twice, discard excess PBS by gently inverting plate and lightly blotting on sterile absorbent paper towel after each wash. Do not allow plates to dry.

2.2.4.3 Transfer 100 µL/well of the virus-serum mixture to the corresponding wells on the A549 cell plate following the plate template (**Figure 4**). Incubate the plate for 1 h at 37 °C, 5% CO_2.

2.2.4.4 After 1 h, using a pipette, take out 90 µL/well and add 100 µL of prewarmed 1x M199 + 1.5% CMC + 2% FCS + pen/strep. Incubate plate for 3 days at 37 °C, 5% CO_2.

NOTE: Make sure the solution is warmed at 37 °C before adding to plates.

2.3 Developing assay plate and analysis (Day 5)

2.3.1 Develop fixation and assay plate following steps 1.3.1.1 to 1.3.1.8.

2.3.2 Determine the 50% neutralization titer

2.3.2.1 Determine the 50% neutralization titer by calculating the proportional distance between the reciprocal serum dilutions above and below 50% of the ‘no serum’ control wells (positive virus control - column 10, rows E-H).

2.3.2.2 Count the number of PFU (*i.e.*, plaques) arising from individual viral infections in serum wells and no serum control wells. Calculate the mean number of PFU of the no serum control wells and determine the 50% value.

2.3.2.3 Identify serum dilutions with counts which are immediately above and below the 50% value of the no serum control wells. Using a semi-log graph or spreadsheet template, plot the number of PFU on the x-axis (linear scale) and the reciprocal serum dilution on the y-axis (log_{10} scale). Draw a line between the two points and read the 50% neutralization titers of the test serum samples from this line.

NOTE: The RSV PRN assay worksheet (**Supplementary Worksheet**) is used to determine the 50% neutralization titer.

REPRESENTATIVE RESULTS:

The titration of a virus stock was performed from 1:10 to $1:10^8$ dilution to determine the virus stock concentration prior to the PRN assay (representative results shown in **Figure 5**). From **Figure 5**, PFU can be counted reliably at dilutions of $1:10^4$ and $1:10^5$. The average number of PFU from triplicate wells at the same dilution was calculated. Since the average number of spots at $1:10^5$ dilution was 14, the viral titer of this viral stock was determined as follows:

14 (average number of spots)/[10^{-5} (dilution) x 0.1 mL (volume of inoculum)] = 1.4×10^7 PFU/mL

Using the above described method, we have measured NAbs for both RSV-A and B in human samples. **Figure 6** demonstrates the interpretation of well images of three representative serum samples with varying NAb titers against RSV-A. **Figure 6A** shows well images of sample titration replicates for each representative serum (the actual assay plate had three technical replicates). Serum dilutions as indicated are different for each sample. Virus control wells containing only virus and no serum had their average PFU calculated and used to determine the 50% neutralization cut-off, which in this example was 52. **Figure 6B** illustrates how the titration of the three sera was determined using this cut-off. The log_2 reciprocal of the serum dilution is plotted on the x-axis and the number of PFU as a percentage of control on the y-axis. Symbol and error bars represent the mean of triplicates ± standard deviation. Fifty percent of the average spots in the virus-control wells (indicated by the horizontal dashed line) was used as a cut-off to determine the 50% neutralizing antibody titer of a serum sample. The neutralizing antibody titer is defined as the reciprocal of the dilution that would result in 50% inhibition of virus activity.

As each experiment includes the RSV reference antiserum and positive virus control wells, monitoring the inter-assay variability provides quality control between experiments. **Figure 7** shows the results of the RSV-A PRN assay in two different adult cohorts from the Gambia cohort (N = 21)[10] and healthy volunteers in Melbourne (N = 36). Titers of RSV NAb were variable within each population, with the Gambian adults having a lower mean NAb titer compared to the Melbourne adults. The RSV reference antiserum is also shown (N = 52 replicates), demonstrating very low variability with a coefficient of variation (CV) of 6.82%.

FIGURE LEGENDS:

Figure 1: Viral titration plate layout for quantitation of RSV stocks. v = stock of RSV virus (v1, stock of viral batch 1; v2, stock of viral batch 2; v3, stock of viral batch 3), N = negative control well, X = media added. Color coding is only to aid visualization of plate layout.

Figure 2: Example count settings used for the spots reader. Screenshot of the parameters typically used for counting virus plaques.

Figure 3: Examples of artefact and disrupted cell monolayers. (**A**) Artefacts. (**B**) Disrupted cell monolayers.

Figure 4: Plate template for RSV PRN assay. V = viral positive control wells, N = negative control wells, X = media added, S = reference antiserum with all dilutions (from 1:100 to 1:12,800) in column 12. Color coding is only to aid visualization of plate layout.

Figure 5: An example of an RSV viral titration result. Representative wells from an assay performed in triplicate are shown. The numbers in each well refer to the number of plaques counted using the spots reader. TMC = too many to count.

Figure 6: Results from a representative RSV PRN assay. (**A**) The number of PFU counted for three individual serum samples according to different dilution ranges. The numbers in each well image refer to the number of plaques counted. (**B**) NAb titer interpretation from the well images in panel **A** for the three representative human serum samples. The data is calculated on the basis of the 50% cut-off from the virus control wells. Horizontal bars represent mean ± SD.

Figure 7: RSV neutralizing antibody titers in Melbourne (N = 21) and Gambian (N = 36) adults. The human RSV reference serum is also shown for comparison (N = 52 replicates). Symbols represent individual titers and horizontal bars represent geometric mean titer ± 95% CI. The N = 52 results are based on three technicians over a two-month period.

Supplementary Figure 1: Comparison of NAb titers for RSV-A. Left: NAb titers calculated using the linear and non-linear regression models (N = 152 assays). **Right:** Correlation between RSV-A NAb titers using both linear and non-linear regression models.

DISCUSSION:

We have developed and optimized a simple and efficient RSV micro-neutralization assay that can be readily adapted in most laboratories. This assay is able to measure viral infection ability as well as measuring the inhibition of viral infection by NAb at the cellular level using computerized image scanning. The use of an imaging-based platform and specific antibody-based systems has increased the specificity and sensitivity of spot detection compared to traditional plaque detection methods[6,7]. This allows characterization of RSV strains with low infectivity and also provides an accurate quantification of RSV concentration prior to use in the PRN assay.

It is recommended that the same batch of viral stock is used for each study in order to minimize any batch-to-batch variations between RSV stocks. Other important aspects of the assay include the consistency of virus input concentration, the integrity of the A549 cell monolayer, and using same count settings to ensure assay consistency and accuracy. The amount of virus added to the plate can be verified again in a separate plaque viral titration. However, monitoring the positive control wells of the PRN assay also provides an indication of actual viral titer and is also worthwhile to monitor across assays. Moreover, including the reference serum in every plate is another quality control measure to ensure that assays are reproducible. Our experiments did not use the recently validated RSV international standard antiserum from the NIBSC, as it was not available at the time of our study[11]. Using this RSV international standard antiserum should be recommended for future studies measuring RSV NAb so that results across laboratories can be compared with confidence.

Development of a relatively simple, high-throughput assay would facilitate global standardization efforts for the use of RSV NAbs as more laboratories worldwide would be able to use this method. This is particularly important given the number of RSV vaccine candidates in development, with some currently in phase 3 trials. It is critical that the results of future clinical studies of RSV vaccines be comparable, and therefore a standardized assay represents a key aspect to this goal. While a number of low- and middle-income countries may not be able to directly support acquisition of the equipment needed, linkages through international collaborations and/or capacity building programs will be critical over the medium to long term.

This NAb assay protocol is flexible in being able to be adapted for different cell lines (A549, Hep2, and Vero) and can be used for different RSV strains. We have also adapted this method for RSV-B successfully. As the assay platform has been developed for a 96-well plate format, this provides a cost-effective and high-throughput alternative with high accuracy because it allows more replicates and the inclusion of the reference serum control and negative control on every plate. Using the same reference serum control is also recommended as this is critical to ensure quality control and quality assurance. In our hands, we observed very low CVs of 6.82% for the reference serum which is much lower than reported CVs for other biological assays in the range of 30-40%[5].

Moreover, the image data and the count data (raw data) generated from this protocol can be stored indefinitely for future reference and/or analysis. Maintaining records of raw data is an essential requirement for clinical trials, particularly those involving future RSV vaccines. Our worksheet (see **Supplementary Worksheet**) suggested in this protocol can help to record all aspects of the experiment for the purpose of quality control and assurance control. The worksheet provides the calculation of the 50% neutralizing titer using a simple linear model as this has the advantage of not requiring any specialized software. However, the raw data from our NAb assay can be analyzed using the non-linear regression model that has also been used in the vaccine research for calculating 50% neutralizing titers, although this requires other software packages[12,13]. Using our experimental data, the results obtained using these two methods were similar, giving confidence to titer values obtained using the simplified linear model (see **Supplementary Figure 1**). The cost for setting up this assay is affordable for many laboratories with perhaps the most expensive item being the spots reader (approximately USD $50K).

However, the spots reader has the advantage of being able to be used for other applications such as the enzyme-linked immunospot (ELISpot) for cytokine and/or antibody-secreting cell measurement, which makes it a valuable component in vaccine trial evaluation.

In conclusion, this improved, high-throughput RSV PRN assay can be easily implemented in many laboratories and will support the international harmonization effort of WHO for RSV NAb assays. This will be critical for the evaluation of novel RSV vaccine candidates in the future.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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	1	2	3	4	5	6	7	8	9	10	11	12
A	v1	v1	v1	v2	v2	v2	v3	v3	v3	N	N	N
B	X	X	X	X	X	X	X	X	X	N	N	N
C	X	X	X	X	X	X	X	X	X	N	N	N
D	X	X	X	X	X	X	X	X	X	N	N	N
E	X	X	X	X	X	X	X	X	X	N	N	N
F	X	X	X	X	X	X	X	X	X	N	N	N
G	X	X	X	X	X	X	X	X	X	N	N	N
H	X	X	X	X	X	X	X	X	X	N	N	N

Figure 2

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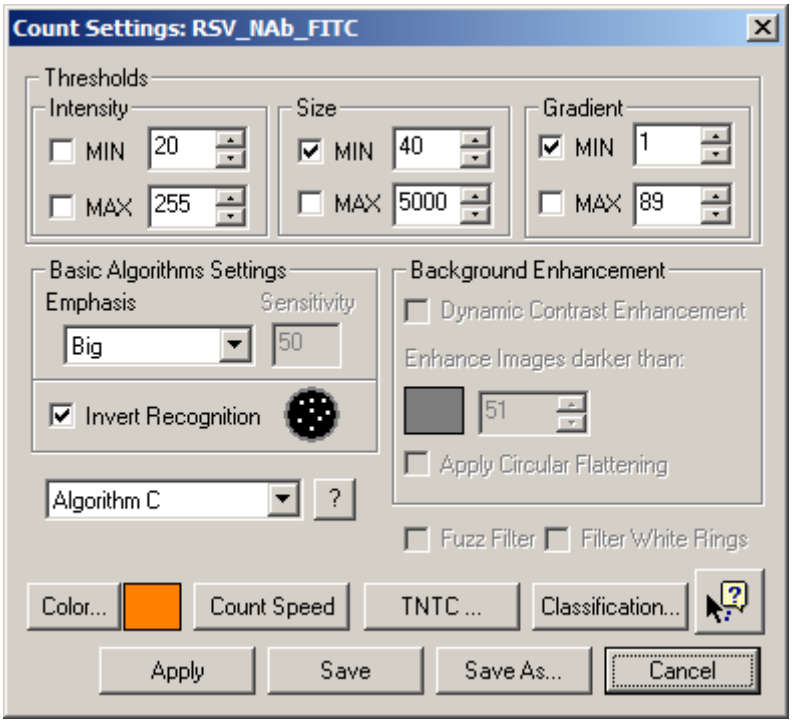

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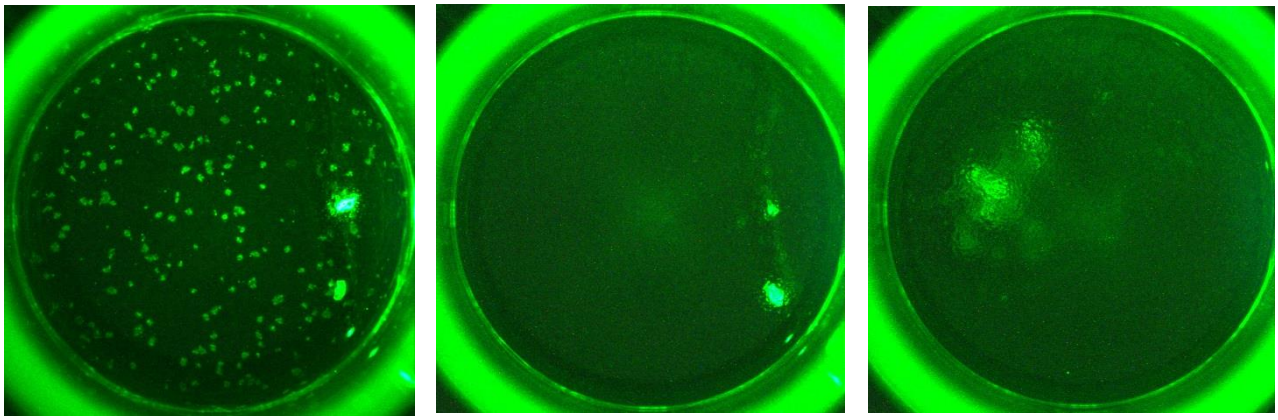
A. Different examples of artefacts



B. Different examples of disrupted cell monolayer

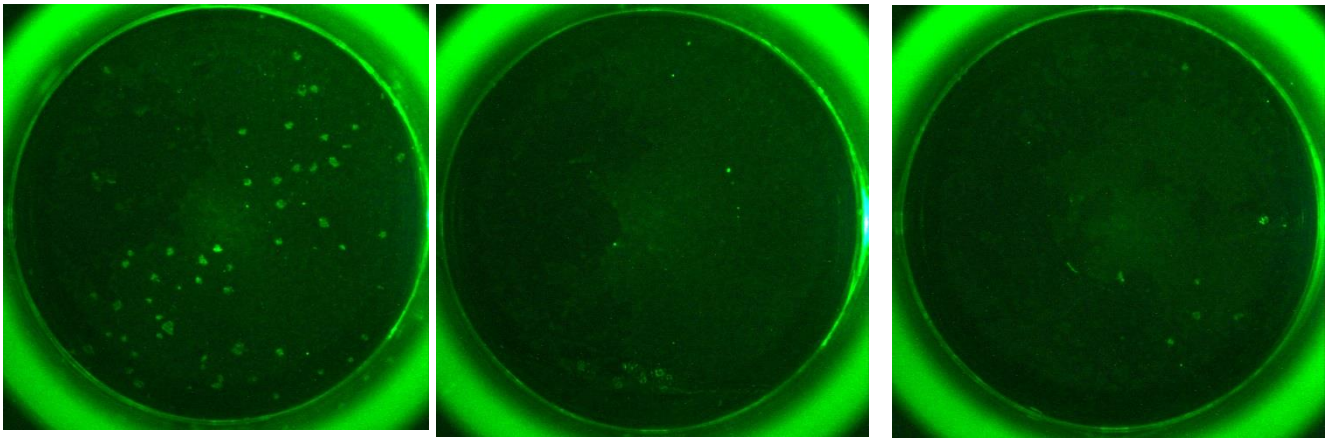


Figure 3: Examples of artefact (A) and disrupted cell monolayers (B)

Figure 4

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	1	2	3	4	5	6	7	8	9	10	11	12
A	sample 1	sample 1	sample 1	sample 2	sample 2	sample 2	sample 3	sample 3	sample 3	N	N	S100
B	x	x	x	x	x	x	x	x	x	N	N	S200
C	x	x	x	x	x	x	x	x	x	N	N	S400
D	x	x	x	x	x	x	x	x	x	N	N	S800
E	sample 4	sample 4	sample 4	sample 5	sample 5	sample 5	sample 6	sample 6	sample 6	V	V	S1600
F	x	x	x	x	x	x	x	x	x	V	V	S3200
G	x	x	x	x	x	x	x	x	x	V	V	S6400
H	x	x	x	x	x	x	x	x	x	V	V	S12800

Figure 5

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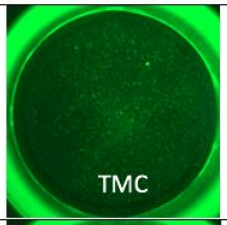
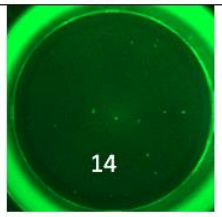
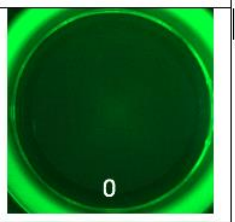
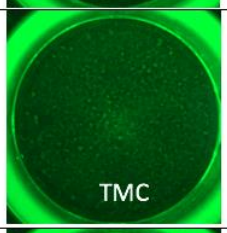
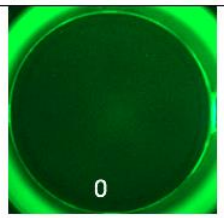
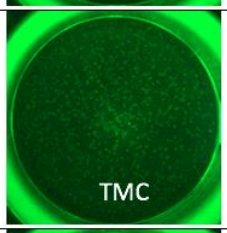
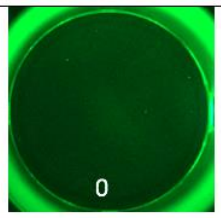
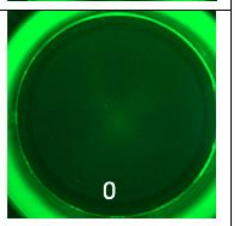
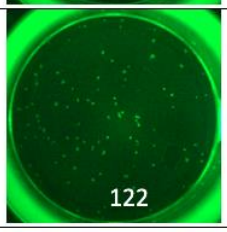
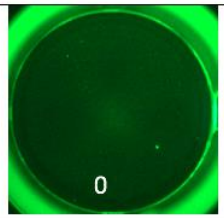
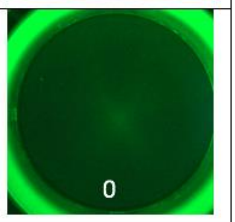
Viral dilution	One replicate	Viral dilution	One replicate	Negative control (NC)	4 replicates
1:10		$1:10^5$		NC	
$1:10^2$		$1:10^6$		NC	
$1:10^3$		$1:10^7$		NC	
$1:10^4$		$1:10^8$		NC	

Figure 6

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A

Representative serum

Serum dilution	Sample 1	Serum dilution	Sample 2	Serum dilution	Sample 3	Virus control Average=104 plaques 50%=52 plaques
1:16	11	1:64	23	1:256	37	100
1:32	38	1:128	40	1:512	45	92
1:64	65	1:256	83	1:1024	59	105
1:128	93	1:512	122	1:2048	75	119

B

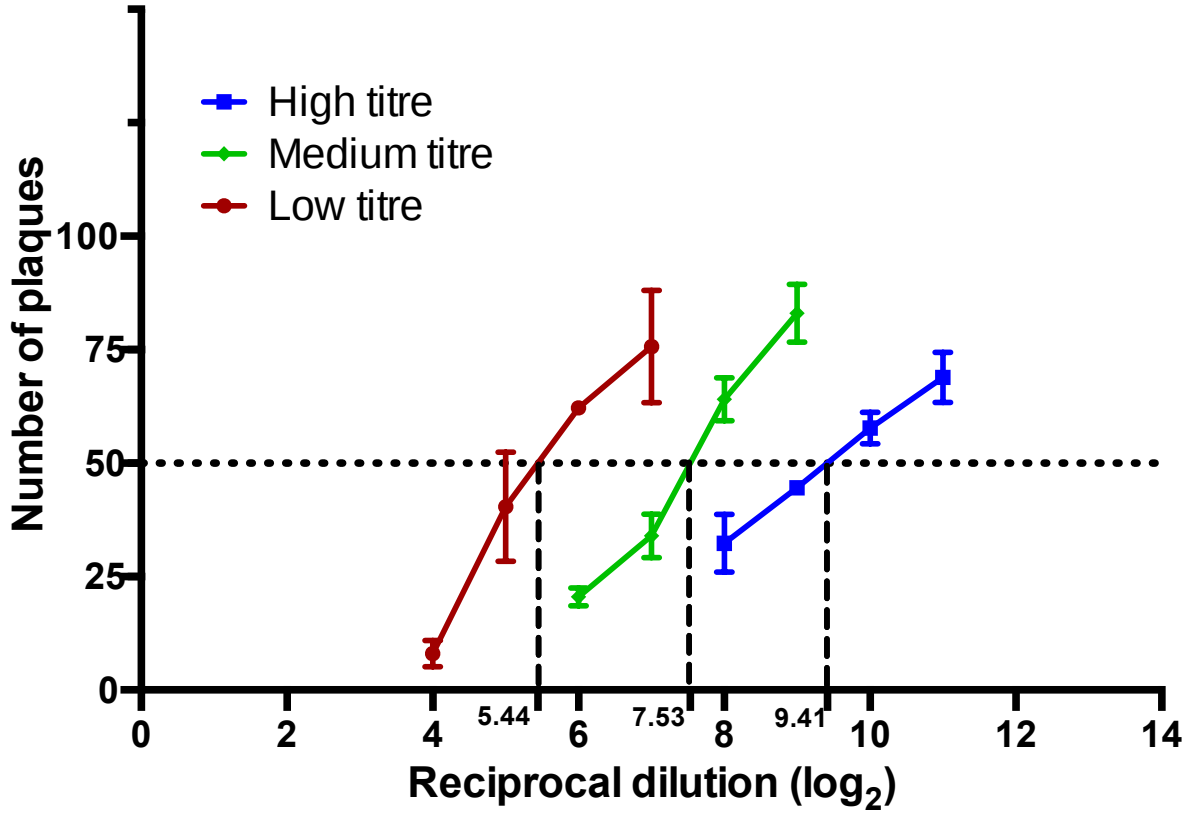

Figure 7

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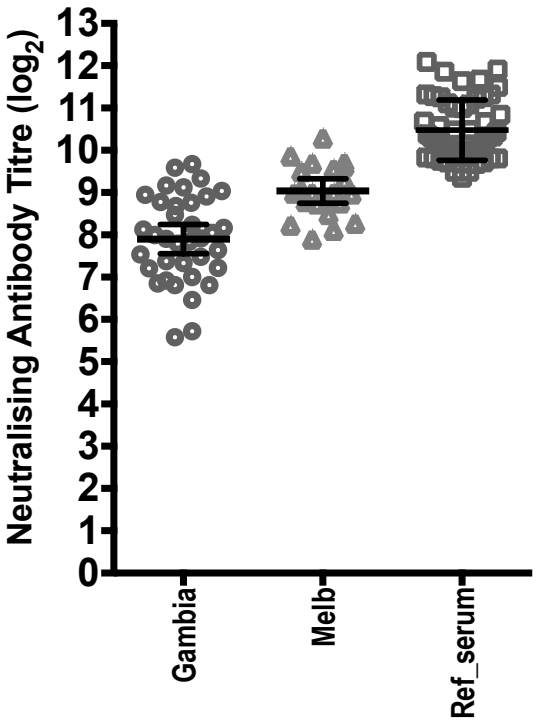

Click here to access/download;Table of Materials;Do et al. JovE Materials Table Revised 021118.xlsx

Name of Reagent/ Equipment	Company
Cell line	
A549	ATCC
Viral strains	
RSV A2	ATCC
Reagents	
Acetone	Merck
Alexa-Fluor donkey anti-goat IgG (stored at 4 °C)	Life Technologies
CMC sodium salt powder	Sigma-Aldrich
DMEM (no serum, 3.7 g/L NaHC, P/S) (stored at 4 °C)	Scientific Services – Tissue Culture
Foetal calf Serum (stored in 50ml aliquots at -20 °C)	Interpath
Goat X RSV antibody	Merck
human polyclonal antiserum to respiratory syncytial virus (RSV) (stored in 45 µL aliquots at -20 °C)	BEI Resources
M199 powder	Life Technologies
Milk diluent blocking solution (stored at 4 °C)	Australian Biosearch
Penicillin/Streptomycin (stored in 6mL aliquots at -20 °C)	Life Technologies
s.d.H_2O from Milli-Q dispenser	Merck
Sterile 1X PBS for culture (stored at 4°C)	Scientific Services – Tissue Culture
Tween 20 polysorbate	Sigma-Aldrich
General Consumables	
Conical Falcon tubes (50 mL)	Invitro Technologies
Filter unit 0.22um (500 mL)	Thermo Fisher
Sterile Eppendorf tubes (1.5 mL)	Australia PL
Sterile flat-bottom plates (96-well with lid)	Interpath
Sterile U-bottom plates (96-well with lid)	Interpath
5ml serological pipette	Sigma-Aldrich
10 mL serological pipette	Interpath
25 mL serological pipette	Sigma-Aldrich
Tip Pipette 1-200 µL Clear Maxymum Recovery Racked Pre-sterilized 10RACKS x 96TIPS PKG960	Fisher Biotec
Tip Pipette 5-20 µL Clear Maxymum Recovery Racked Pre-sterilized 10RACKS x 96TIPS PKG960	Fisher Biotec
Tip Pipette 100-1000 µLClear Maxymum Recovery Racked Pre-sterilized 10RACKS x 100TIPS PKG1000	Fisher Biotec
Tip Pipette 1-10 µL Clear Maxymum Recovery Racked Pre-sterilized 10RACKS x 100TIPS PKG1001	Fisher Biotec
Equipments and softwares	

ELISpot reader system	AID iSpot, Autoimmun Diag
AID ELISpot software version 5.0	AID iSpot, Autoimmun Diag
Microsoft Excel 2007	

Catalog Number	Comments/Description
CCL-185	provided by Dr Keith Chappell, University of Queensland
VR-1540	lot number 60430286
1000142511	
A11055	
C5678-500G	
	MCRI in house supply
SFBS-F	
AB1128	
NR-4022	Free order through BEI Resources upon registration. This serum belong to a panel of human antiserum and immune globulin to RSV (NR-32832)
31100035	
50-82-01	
15140122	
	In-house dispensation
	MCRI in house supply
9005-64-5	
FAL352070	
NAL5660020	
AM12400	
655180	
650180	
CLS4487-200EA	
607180	
CLS4251-200EA	
TF-200-L-R-S	
TF-20-L-R-S	
TF-1000-L-R-S	
TXLF-10-L-R-S	

gnostika GmbH, Strasburg, Germany
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Author(s): Lien Anh Ha Do, Reuben Tee Jun Le, Nathaniel Jeremy Anderson, Dorin Sruyanujaya Ng, Kevin Chappell, Kim Mulholland, Paul V Licciardi.

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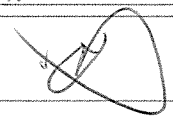
Signature:	
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Rebuttal Letter
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Prof Xiaoyan Cao
Review Associate
Journal of Visualized Experiments
USA

2nd November 2018

Dear Prof Cao,

We have provided our responses to the editorial comments below as well as in the manuscript as Tracked Changes.

Editorial comments:

1. *As we can only film 2.75 pages of the protocol, please review and shorten the highlighted portion to 2.75 pages. Note that the highlighted content should be continuous and contain essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please note that the editor has made some minor changes (e.g., combining some shorter steps) to condense the highlighted content.*

Thank-you for your help with this. We have made some additional modifications to the highlighted sections of the protocol so that it is approximately 2.75 pages in length.

2. *Please address specific comments marked in the attached manuscript.*

We have addressed the specific comments marked in the revised manuscript.

Thank-you for considering our revised manuscript for publication in JoVE.

Yours sincerely,

A/Prof Paul Licciardi
Murdoch Children's Research Institute
Melbourne, Australia

Supplemental Worksheet

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Experiment starting: *dd/mm/yyyy*
D1: Cell plate prepared by (initials)
D2: Performed by (initials) *XY*
D5: Performed by (initials)

Plate 1

		1	2	3	4	5	6	7	8	9	10	11	12
Samples	*study name*	A	sample name			sample name			sample name			N	N	Ref
		B	x	x	x	x	x	x	x	x	x	N	N	S200
		C	x	x	x	x	x	x	x	x	x	N	N	S400
		D	x	x	x	x	x	x	x	x	x	N	N	S800
Virus stock	*stock (concentration pfu/ml)*	E	sample name			sample name			sample name			V	V	S1600
Final dilution	*dilution (concentration pfu/ml)*	F	x	x	x	x	x	x	x	x	x	V	V	S3200
		G	x	x	x	x	x	x	x	x	x	V	V	S6400
		H	x	x	x	x	x	x	x	x	x	V	V	S12800

Serial dilution *XX* µL stock + *XX* µL media (1/*XX*)
(Final = 1/*XX*) *XX* µL dilution + *XX* µL media (1/*X* X)

Primary antibody: Gt X RSV antibody / AB1128 (1:500)
Secondary antibody: Alexa Fluor 488 Donkey Anti-goat IgG H+L 0.5ML / A11055 (1:5000)

	Sample	**Date**	**Starting dilution (1/X)**
Sample 1	*sample name*	*dd/mm/yy*	*XX*
Sample 2	*sample name*	*dd/mm/yy*	*XX*
Sample 3	*sample name*	*dd/mm/yy*	*XX*
Sample 4	*sample name*	*dd/mm/yy*	*XX*
Sample 5	*sample name*	*dd/mm/yy*	*XX*
Sample 6	*sample name*	*dd/mm/yy*	*XX*

Experiment starting: *dd/mm/yyyy*
D1: Cell plate prepared by (initials)
D2: Performed by (initials) *XY*
D5: Performed by (initials)

Plate 2

		1	2	3	4	5	6	7	8	9	10	11	12
Samples	*study name*	A	sample name			sample name			sample name			N	N	Ref
		B	x	x	x	x	x	x	x	x	x	N	N	S200
		C	x	x	x	x	x	x	x	x	x	N	N	S400
		D	x	x	x	x	x	x	x	x	x	N	N	S800
Virus stock	*stock (concentration pfu/ml)*	E	sample name			sample name			sample name			V	V	S1600
Final dilution	*dilution (concentration pfu/ml)*	F	x	x	x	x	x	x	x	x	x	V	V	S3200
		G	x	x	x	x	x	x	x	x	x	V	V	S6400
		H	x	x	x	x	x	x	x	x	x	V	V	S12800

Serial dilution XX µL stock + XX µL media (1/XX)
(Final = 1/XX) XX µL dilution + XX µL media (1/XX)

Primary antibody: Gt X RSV antibody / AB1128 (1:500)
Secondary antibody: Alexa Fluor 488 Donkey Anti-goat IgG H+L 0.5ML / A11055 (1:2500)

	Sample	**Date**	**Starting dilution (1/X)**
Sample 7	*sample name*	*dd/mm/yy*	*XX*
Sample 8	*sample name*	*dd/mm/yy*	*XX*
Sample 9	*sample name*	*dd/mm/yy*	*XX*
Sample 10	*sample name*	*dd/mm/yy*	*XX*
Sample 11	*sample name*	*dd/mm/yy*	*XX*
Sample 12	*sample name*	*dd/mm/yy*	*XX*

Experiment starting: *dd/mm/yyyy*
D1: Cell plate prepared by (initials)
D2: Performed by (initials) *XY*
D5: Performed by (initials)

Plate 3

		1	2	3	4	5	6	7	8	9	10	11	12
Samples	*study name*	A	sample name			sample name			sample name			N	N	Ref
Performed by (initials)	*XY*	B	x	x	x	x	x	x	x	x	x	N	N	S200
		C	x	x	x	x	x	x	x	x	x	N	N	S400
		D	x	x	x	x	x	x	x	x	x	N	N	S800
Virus stock	*stock (concentration pfu/ml)*	E	sample name			sample name			sample name			V	V	S1600
Final dilution	*dilution (concentration pfu/ml)*	F	x	x	x	x	x	x	x	x	x	V	V	S3200
		G	x	x	x	x	x	x	x	x	x	V	V	S6400
		H	x	x	x	x	x	x	x	x	x	V	V	S12800

Serial dilution XX µL stock + XX µL media (1/XX)
(Final = 1/XX) XX µL dilution + XX µL media (1/XX)

Primary antibody: Gt X RSV antibody / AB1128 (1:500)
Secondary antibody: Alexa Fluor 488 Donkey Anti-goat IgG H+L 0.5ML / A11055 (1:2500)

	Sample	**Date**	**Starting dilution (1/X)**
Sample 13	*sample name*	*dd/mm/yy*	*XX*
Sample 14	*sample name*	*dd/mm/yy*	*XX*
Sample 15	*sample name*	*dd/mm/yy*	*XX*
Sample 16	*sample name*	*dd/mm/yy*	*XX*
Sample 17	*sample name*	*dd/mm/yy*	*XX*
Sample 18	*sample name*	*dd/mm/yy*	*XX*

Experiment starting: *dd/mm/yyyy*
D1: Cell plate prepared by (initials)
D2: Performed by (initials) *XY*
D5: Performed by (initials)

Plate 4

		1	2	3	4	5	6	7	8	9	10	11	12
Samples	*study name*	A	sample name			sample name			sample name			N	N	Ref
Performed by (initials)	*XY*	B	x	x	x	x	x	x	x	x	x	N	N	S200
		C	x	x	x	x	x	x	x	x	x	N	N	S400
		D	x	x	x	x	x	x	x	x	x	N	N	S800
Virus stock	*stock (concentration pfu/ml)*	E	sample name			sample name			sample name			V	V	S1600
Final dilution	*dilution (concentration pfu/ml)*	F	x	x	x	x	x	x	x	x	x	V	V	S3200
		G	x	x	x	x	x	x	x	x	x	V	V	S6400
		H	x	x	x	x	x	x	x	x	x	V	V	S12800

Serial dilution XX µL stock + XX µL media (1/XX)
(Final = 1/XX) XX µL dilution + XX µL media (1/XX)

Primary antibody: Gt X RSV antibody / AB1128 (1:500)
Secondary antibody: Alexa Fluor 488 Donkey Anti-goat IgG H+L 0.5ML / A11055 (1:2500)

	Sample	**Date**	**Starting dilution (1/X)**
Sample 19	*sample name*	*dd/mm/yy*	*XX*
Sample 20	*sample name*	*dd/mm/yy*	*XX*
Sample 21	*sample name*	*dd/mm/yy*	*XX*
Sample 22	*sample name*	*dd/mm/yy*	*XX*
Sample 23	*sample name*	*dd/mm/yy*	*XX*
Sample 24	*sample name*	*dd/mm/yy*	*XX*

#Paste your couting result to this table

	1	2	3	4	5	6	7	8	9	10	11	12	Ref Dilut.
A													100
B													200
C													400
D													800
E													1600
F													3200
G													6400
H													12800

Pos cont	#DIV/0!
50%	#DIV/0!

Sample 1	Dilution (1/x)
#DIV/0!	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 2	Dilution (1/x)
#DIV/0!	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 3	Dilution (1/x)
#DIV/0!	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 4	Dilution (1/x)
#DIV/0!	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 5	Dilution (1/x)
#DIV/0!	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 6	Dilution (1/x)
#DIV/0!	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Mean count	Dilution
xx	*xx*
xx	*xx*
50% titre	#DIV/0!
50% titre (log	#DIV/0!

Mean count	Dilution
xx	*xx*
xx	*xx*
50% titre	#DIV/0!
50% titre (lo	#DIV/0!

Mean count	Dilution
xx	*xx*
xx	*xx*
50% titre	#DIV/0!
50% titre (log	#DIV/0!

Mean count	Dilution
xx	*xx*
xx	*xx*
50% titre	#DIV/0!
50% titre (log	#DIV/0!

Mean count	Dilution
xx	*xx*
xx	*xx*
50% titre	#DIV/0!
50% titre (lo	#DIV/0!

Mean count	Dilution
xx	*xx*
xx	*xx*
50% titre	#DIV/0!
50% titre (lo	#DIV/0!

	Sample	Date	Dilution	50% titre (log2)
Sample 1	sample name	dd/mm/yy	XX	#DIV/0!
Sample 2	sample name	dd/mm/yy	XX	#DIV/0!
Sample 3	sample name	dd/mm/yy	XX	#DIV/0!
Sample 4	sample name	dd/mm/yy	XX	#DIV/0!
Sample 5	sample name	dd/mm/yy	XX	#DIV/0!
Sample 6	sample name	dd/mm/yy	XX	#DIV/0!

Reference	Mean count	Dilution
below 50%	*xx*	*xx*
above 50%	*xx*	*xx*
50% titre	#DIV/0!	
50% titre (lo	#DIV/0!	

#Paste your couting result to this table

	1	2	3	4	5	6	7	8	9	10	11	12	Ref Dilut.
A													100
B													200
C													400
D													800
E													1600
F													3200
G													6400
H													12800

Pos cont	#DIV/0!
50%	#DIV/0!

Sample 7	Dilution (1/x)
#DIV/0!	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 8	Dilution (1/x)
#DIV/0!	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 9	Dilution (1/x)
#DIV/0!	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 10	Dilution (1/x)
#DIV/0!	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 11	Dilution (1/x)
#DIV/0!	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 12	Dilution (1/x)
#DIV/0!	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Mean count	Dilution
xx	*xx*
xx	*xx*
50% titre	#DIV/0!
50% titre (log	#DIV/0!

Mean count	Dilution
xx	*xx*
xx	*xx*
50% titre	#DIV/0!
50% titre (lo	#DIV/0!

Mean count	Dilution
xx	*xx*
xx	*xx*
50% titre	#DIV/0!
50% titre (log	#DIV/0!

Mean count	Dilution
xx	*xx*
xx	*xx*
50% titre	#DIV/0!
50% titre (log	#DIV/0!

Mean count	Dilution
xx	*xx*
xx	*xx*
50% titre	#DIV/0!
50% titre (lo	#DIV/0!

Mean count	Dilution
xx	*xx*
xx	*xx*
50% titre	#DIV/0!
50% titre (lo	#DIV/0!

	Sample	Date	Dilution	50% titre (log2)
Sample 7	sample name	dd/mm/yy	XX	#DIV/0!
Sample 8	sample name	dd/mm/yy	XX	#DIV/0!
Sample 9	sample name	dd/mm/yy	XX	#DIV/0!
Sample 10	sample name	dd/mm/yy	XX	#DIV/0!
Sample 11	sample name	dd/mm/yy	XX	#DIV/0!
Sample 12	sample name	dd/mm/yy	XX	#DIV/0!

Reference	Mean count	Dilution
below 50%	*xx*	*xx*
above 50%	*xx*	*xx*
50% titre	#DIV/0!	
50% titre (lo	#DIV/0!	

#Paste your couting result to this table

	1	2	3	4	5	6	7	8	9	10	11	12	Ref Dilut.
A													100
B													200
C													400
D													800
E													1600
F													3200

G		6400
H		12800
	50%	0

Sample 13	Dilution (1/x)
2	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 14	Dilution (1/x)
5	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 15	Dilution (1/x)
8	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 16	Dilution (1/x)
#DIV/0!	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 17	Dilution (1/x)
#DIV/0!	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 18	Dilution (1/x)
#DIV/0!	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Mean count	Dilution
XX	*XX*
XX	*XX*
50% titre	#DIV/0!
50% titre (log	#DIV/0!

Mean count	Dilution
XX	*XX*
XX	*XX*
50% titre	#DIV/0!
50% titre (lo	#DIV/0!

Mean count	Dilution
XX	*XX*
XX	*XX*
50% titre	#DIV/0!
50% titre (lo	#DIV/0!

Mean count	Dilution
XX	*XX*
XX	*XX*
50% titre	#DIV/0!
50% titre (log	#DIV/0!

Mean count	Dilution
XX	*XX*
XX	*XX*
50% titre	#DIV/0!
50% titre (lo	#DIV/0!

Mean count	Dilution
XX	*XX*
XX	*XX*
50% titre	#DIV/0!
50% titre (lo	#DIV/0!

	Sample	Date	Dilution	50% titre (log2)
Sample 13	sample name	dd/mm/yy	XX	#DIV/0!
Sample 14	sample name	dd/mm/yy	XX	#DIV/0!
Sample 15	sample name	dd/mm/yy	XX	#DIV/0!
Sample 16	sample name	dd/mm/yy	XX	#DIV/0!
Sample 17	sample name	dd/mm/yy	XX	#DIV/0!
Sample 18	sample name	dd/mm/yy	XX	#DIV/0!

Reference	Mean count	Dilution
below 50%	*XX*	*XX*
above 50%	*XX*	*XX*
50% titre	#DIV/0!	
50% titre (lo	#DIV/0!	

#Paste your couting result to this table

	1	2	3	4	5	6	7	8	9	10	11	12	Ref Dilut.
A													100
B													200
C													400
D													800
E													1600
F													3200
G													6400
H													12800
									50%	0			

Sample 19	Dilution (1/x)
2	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 20	Dilution (1/x)
5	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 21	Dilution (1/x)
8	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 22	Dilution (1/x)
#DIV/0!	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 23	Dilution (1/x)
#DIV/0!	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Sample 24	Dilution (1/x)
#DIV/0!	XX
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!
#DIV/0!	#VALUE!

Mean count	Dilution
XX	*XX*
XX	*XX*
50% titre	#DIV/0!
50% titre (log	#DIV/0!

Mean count	Dilution
XX	*XX*
XX	*XX*
50% titre	#DIV/0!
50% titre (lo	#DIV/0!

Mean count	Dilution
XX	*XX*
XX	*XX*
50% titre	#DIV/0!
50% titre (lo	#DIV/0!

Mean count	Dilution
XX	*XX*
XX	*XX*
50% titre	#DIV/0!
50% titre (log	#DIV/0!

Mean count	Dilution
XX	*XX*
XX	*XX*
50% titre	#DIV/0!
50% titre (lo	#DIV/0!

Mean count	Dilution
XX	*XX*
XX	*XX*
50% titre	#DIV/0!
50% titre (lo	#DIV/0!

	Sample	Date	Dilution	50% titre (log2)
Sample 19	sample name	dd/mm/yy	XX	#DIV/0!
Sample 20	sample name	dd/mm/yy	XX	#DIV/0!
Sample 21	sample name	dd/mm/yy	XX	#DIV/0!
Sample 22	sample name	dd/mm/yy	XX	#DIV/0!
Sample 23	sample name	dd/mm/yy	XX	#DIV/0!
Sample 24	sample name	dd/mm/yy	XX	#DIV/0!

Reference	Mean count	Dilution
below 50%	*XX*	*XX*
above 50%	*XX*	*XX*
50% titre	#DIV/0!	
50% titre (lo	#DIV/0!	

Supplemental Figure

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Do et al. Supplementary Figure 1 Revised