Development and application of a higher throughput RSV plaque assay by immunofluorescent imaging

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A B S T R A C T

Viral plaque assays are important tools in the development and evaluation of new antiviral drugs or vaccines in both preclinical and clinical research. While plaque assays are the standard tools to measure infectious virus, the methodology is time-consuming and requires experience in recognizing plaques. The assays are also prone to variation among analysts due to plaque recognition and manual counting errors. Here we describe the development of two simplified plaque assays for measuring RSV virus titers and anti-RSV antibody neutralization titers using 96 well plate formats. First, we evaluated multiple parameters to build up a quantitative plaque assay to measure infectious RSV. We then optimized the assay conditions to assess the fundamental changes from the traditional plaque assay, which were elimination of overnight pre-seeding host cells and addition of a centrifugation step after viral infection of the cells. We designed DoE to refine four key parameters within one experiment for host cell density, host cell volume, viral inoculum volume, host cell and viral mixture incubation time to make this assay more robust. We have also adapted these conditions into a second assay, which was an automated plaque reduction neutralization assay (PRNT) to determine neutralization titers of anti-RSV antibodies. Both assays utilize immune fluorescence staining to detect viral plaques. The images of the immunostained wells are captured by the PerkinElmer EnSight instrument and show clear visualization of plaques harvesting on day 3. Software algorithm was specifically designed for automatic counting of these fluorescent "objects". The quantitative plaque assay provided titers of RSV similar to those obtained from the traditional plaque assay. The method has been successfully utilized to screen multiple vaccine candidates in viral shedding efficacy studies. The automated PRNT assay provided antibody neutralizing titers that matched with published data. This automated 96 well plaque assay has made it possible to screen RSV samples in a higher throughput manner, and can be extended to other infectious organisms that form plaques for vaccine or drug evaluation.

1. Introduction

In the virology field, plaque assays have been the gold standard for quantitation of viral titers for plaque forming viruses. Since the classic plaque assay was first described by Dulbecco for an animal virus in 1952 and for poliovirus in 1954 (Dulbecco, 1952; Dulbecco and Vogt., 1954), many attempts have been reported to improve or adapt this assay for specific viral organisms. For respiratory syncytial virus (RSV), the plaque assay is normally performed in a 6–24 well plate, and plaques can be manually counted under microscopy in 9–13 days under agar or liquid overlays (Kisch and Johnson, 1963; Loh et al., 1977). Shorter plaque forming time, typically 5–7 days, was also reported under a semi-fluid overlay of methylcellulose (Coates et al., 1966; Trépanier et al., 1980). Colorimetric dyes such as crystal violent or

neutral red are commonly used for staining the host cells to visualize the plaques (Power et al., 1997; Feldman et al., 2001; McKimm-Breschkin, 2004; Rameix-Welti, 2014). This requires a long incubation time to allow the plaques to become large enough so that they can be visualized and counted by eye or microscopically since plaques formed by RSV tend to be small. Though two days of incubation time and specific antibody staining for visualizing and counting RSV plaque with naked eye or under microscopy has been reported (Kyung Sook Kim et al.,2017), the infectious CPE is foci like, no lytic central hole has shown up and the virus is not forming real plaque yet at this time point.

Regardless of the aforementioned efforts to improve the RSV plaque assay, the assay is still time consuming, not amenable to a large number of samples, and difficult to adapt to a high throughput manner. Furthermore, the assay tends to have large variations among different

analysts due to plaque recognition and manual counting errors. Therefore, higher throughput assays, which would limit the bias among analysts, and provide more rapid and accurate counting for the measurement of viral titers, are needed. Here we report the development of two simplified plaque assays for measuring RSV virus titers and anti-RSV antibody neutralization titers using 96 well plate formats. First, we developed a 96 well quantitative plaque assay to measure infectious RSV. To build up this assay, we evaluated multiple parameters such as initial viral inoculum, plate washing methods, infection time, HEp-2 host cell concentration, medium selection, detection antibody screening, overnight pre-seeding of host cells elimination and a centrifugation step addition. We further optimized the assay conditions by running different levels of virus input that targeted different viral plaque forming units, either with or without pre-seeding of cells or with and without the centrifugation step. We designed DoE to make this assay more robust by refining four key parameters within one experiment for host cell density, host cell volume, viral inoculum volume, host cell and viral mixture incubation time. We have also adapted the assay conditions into a second assay platform, which was an automated plaque reduction neutralization assay (PRNT) to determine antibody neutralization endpoint titers. Both assays utilize immune fluorescence staining to detect viral plaques. The images of the immunostained wells are captured by the PerkinElmer EnSight instrument and show clear visualization of plaques harvesting on day 3. Software algorithm was specifically designed for automatic counting of these fluorescent "objects". Though on day 2, anti-RSV antibody stained infectious CPE can be counted (Kyung Sook Kim et al.,2017), they can be easily confused with tissue debris from animal lung and nose samples or "hair like" components from medium (Supplemental Fig. 1). Therefore, we chose day 3 for clear lytic plaque visualization and counting, which were more specific and had lower background.

The quantitative plaque assay provided titers of RSV similar to those obtained from the traditional plaque assay. The method has been successfully utilized to screen multiple vaccine candidates in viral shedding efficacy studies. The automated PRNT assay provided antibodies neutralizing titers that matched with published data. To our knowledge, this is the first time that higher throughput assays with automated plaque detection are reported and used as tools to evaluate viral vaccine or drug candidates.

2. Materials and methods

2.1. Cells and virus

Host HEp-2 cells (ATCC) were propagated twice per week in EMEM (Bio Whittaker) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS, HyClone) and antibiotics (streptomycin, 100 mg/ml and penicillin, 100 U/ml). Virus stocks of RSV strain A Long, A2, and strain B Washington were generated by infecting HEp-2 cells following standard infection and harvesting procedures (Kyung Sook Kim et al., 2017).

2.2. Standard 24 well plaque assay

In order to compare with the 96 well plaque assay, the standard 24 well plaque assay was performed as previously described (McKimm-Breschkin, 2004) with the modification of crystal violet staining replacing the neutral red staining.

2.3. 96 well plaque assay

HEp-2 cells at a concentration of 0.8×10^6 to 1.2×10^6 cells/ml in EMEM medium (EMEM supplemented with 2% FBS and 2 mM glutamine) were added at 50 μl per well into Poly-D coated 96 well flat bottom plates (Corning). Antibodies and serum samples were tested in duplicate using 10 of 2 or 3 fold serial dilutions, mixed 1:1 with the

virus inoculum using 75ul of each prior to adding cells, and incubating for 1 h at 37C in 5% CO2. Cells were added to the sample and virus mixtures, and then incubated for 1 h at 37 °C before the plates were centrifuged at $300 \times g$ for 10 min. 150 μl of EMEM medium supplemented with 1% methylcellulose was overlaid in each well. Plates were incubated at 37 °C with 5% CO2 for 3 days. Cells were then fixed with ice-cold 80% acetone (Sigma) for 10–20 minutes. The plates were allowed to dry for 20 min before washing with PBS supplemented with 0.05% Tween-20 (PBST). Fixed cells were stained with an either a mixture of an in-house generated mouse anti-F monoclonal antibody (1.25 μg/ml) and an in-house generated mouse anti-N (nucleoprotein) monoclonal antibody (1.25 μg/ml) or a rabbit anti-F monoclonal antibody (Sino Biologics). Primary antibodies were incubated with the fixed cells for 1 h before anti-mouse or anti-rabbit IgG Alex488 conjugated secondary antibodies (Invitrogen) was added (1:500 diluted). Unbound secondary antibodies were washed off following 1 h of incubation. Plates were analyzed for image capturing and automated counting by EnSight imager reader 2.02 (PerkinElmer). Virus titers were calculated based on counts and expressed as pfu/ml.

2.4. Establishment, optimization and qualification of the 96 well plaque assay

To build up the 96 well quantitative plaque assay, multiple parameters were tested including initial viral inoculum from 10ul to 40ul with virus concentration at 100 pfu/ml; plate washing methods either by hand or by a plate washer machine; infection time of Day 3 or Day 4; initial HEp-2 host cell concentration from 4×10^5 to 8×10^5 cells per ml; medium evaluation; detection antibody screening; and elimination of pre-seeding of host cells and the addition of a centrifugation step. For detection antibody screen, four of commercially obtained mouse monoclonal antibodies (EMD) along with in-house generated mouse monoclonal antibodies and a rabbit monoclonal antibody 11049-R009 (Sino Biologics) were tested. For EMD antibodies, anti-F MAB 1 referred to MAB858-1, clone 133-1 H; MAB 2 referred to MAB858 -2, clone 131-2 G; MAB 3 referred to MAB858 -3, clone 131-12 H; MAB 3 F referred to MAB858 -3, clone 131-12H conjugated with FITC fluoresces dye. To evaluate two fundamental changes in this assay, we ran the 96 well assays with n = 24 replicates at three different levels of input virus that targeted viral plaque forming unit at 20, 8 or 4 pfu/well, either with or without pre-seeding of cells or with and without the centrifugation step.

DoE was designed and performed to meet or exceed assay sensitivity of the traditional 24 well plaque assay. Four parameters were refined within one experiment: host cell density, host cell volume, viral inoculum volume, host cell and viral mixture incubation time. We evaluated the following conditions: host cell density at 2E4, 4E4 and 6E4 per well; cell volume at 20 μl, 50 μl and 100 μl per well; virus and host cell incubation time at 1, 2 and 4 h; and virus inoculum at 20 μl, 75 μl and 150 μl per well using a virus concentration of 6.7 pfu/ml. 6.7 pfu/ml was the virus input concentration to meet the limit of detection for the 24 well plaque assay.

2.5. Generation and robustness of automated counting algorithm

2.5.1. Generation of algorithm

Automated counting algorithm was generated by PerkinElmer based on images of plaques captured by the EnSight imager (patent submitted, EP20160171730). The imager contain bright field and fluorescence image acquisition capacities. Counting algorithm is mainly based on shape and size of the plaque, fluorescence intensity and diameter of the lytic hole in the cell monolayer. Bright field captures the holes in host cell monolayer including the holes caused by not confluent or damaged cells. The fluorescence imager captures fluorescent intensity representing the real lytic holes and background staining that occurs sometimes for tissue samples, such as lung and nose samples. Combined bright field and fluorescence detections define a real plaque. Young
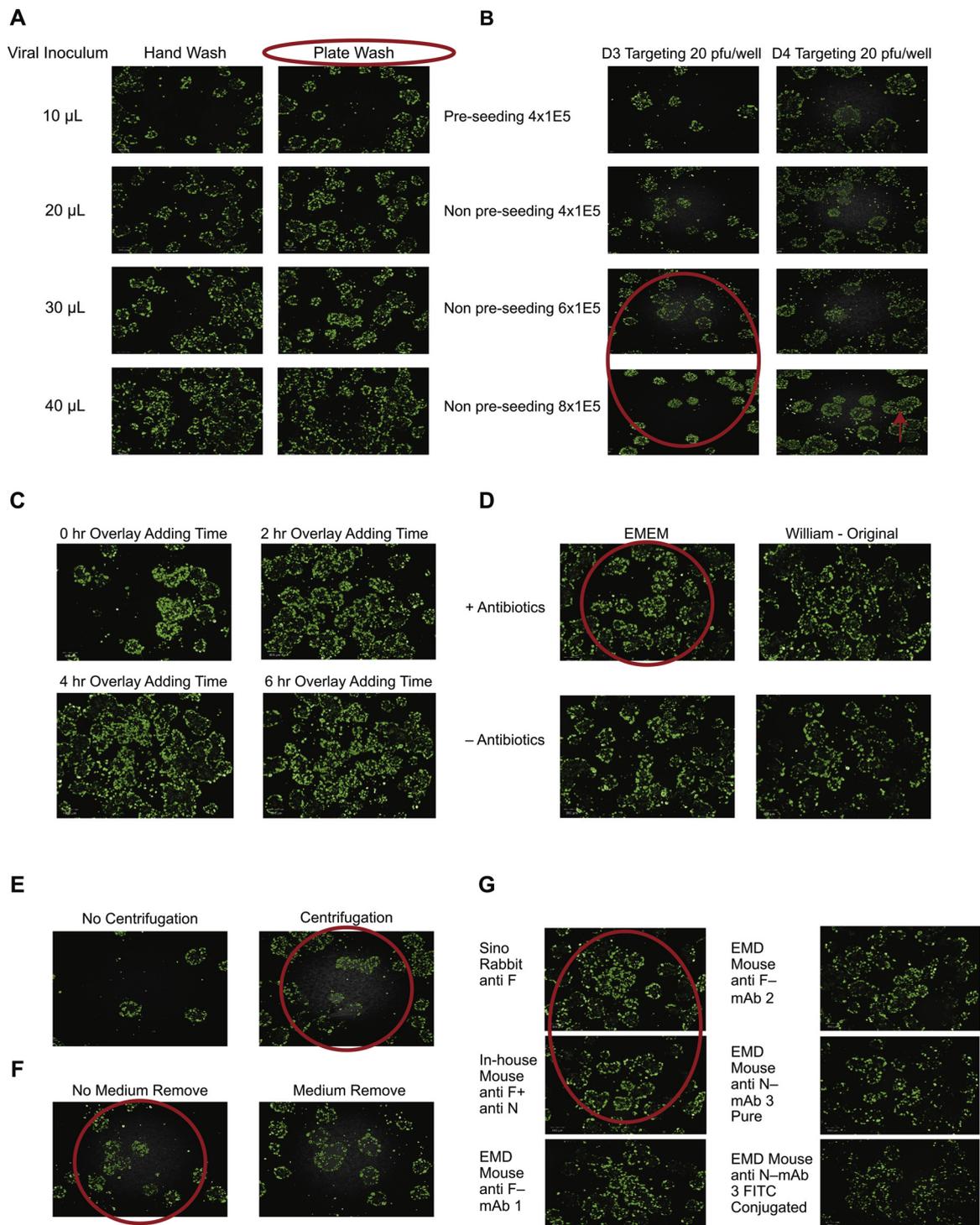
Fig. 1. Establishment of a 96 well plaque assay. Parameters evaluated including initial viral inoculum (A); plate washing methods (A); HEp-2 host cell concentration (B); infection time (B); modification from pre-seeding the host cells to non pre-seeding method (B) initial incubation time survey for mixture of cells and virus prior to add overlay, this condition was further defined in later DoE design stage (C); medium evaluation (D); addition of a centrifugation step (E); medium removal prior to addition of the overlay (F) and detection antibody screening; (G). The red circles indicate the conditions that have been adapted at this development stage. An arrow in Fig. 1B indicates a merged plaque from day 4 incubation (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

plaques that not yet form lytic holes are determined by the intensity of fluorescence, cell texture threshold and size of the plaques. The parameters defined in the algorithm are adjustable based on organism specificity. The algorithm counts plaques in all wells across all dilutions. The plaque counts that are smaller than 100 plaques per well and fit in the linear curve of the dilution range are used to determine the

infectious titers. The counting algorithm was built into the EnSight software Kaleido, and was named as "Virus Plaque Analysis for Kaleido 2.02".

2.5.2. Robustness of algorithm

We compared plaque counting with an EnSight PE algorithm to

manual counting of RSV plaques by three individual analysts. Total of 1920 wells from optimizing viral infection time experiments described in Section 2.4 (Fig. 1B) were counted by each analyst to verify the accuracy of the automated counting algorithm.

2.6. Cotton rat immunization and challenge study

Briefly, four to eight week old female cotton rats (*Sigmodon hispidus*, n = 6) were immunized intramuscularly (IM) with three dose levels (0.1 mcg, 1 mcg or 10 mcg) of DS-Cav1 protein (McLellan et al., 2013) with aluminium phosphate adjuvants on D0 and D28. WT RSV A2 virus was intranasally inoculated on D0 as a positive control. Animals were intranasally challenged with RSV A2 strain at 10^5.5 pfu on D56. Lung and nose tissues were harvested and homogenized on D60. A control group consisted of a challenged cohort of non-vaccinated animals. Lung and nose samples were clarified by centrifugation at 2000 rpm for 10 min, aliquoted, flash frozen, and immediately stored frozen at −70 °C. The animal studies were approved by the Institutional Animal Care and Use Committee of Merck & Co., Inc., Kenilworth, NJ, USA, and conducted in accordance with animal care guidelines.

2.7. Plaque reduction neutralization assay

In a 96 well plaque reduction neutralization assay, monoclonal antibody samples in duplicate were used at 10 ug/ml as the starting concentration, ten of two or three fold serial diluted, and applied 50 μl into each well. 50 μl of diluted virus at 100 pfu/well were added and mixed with samples. The sample and virus mixtures were incubated for 1 h, then 25 μl of HEp-2 host cells at concentration of 1.2×10^6 cells per ml were added to each well. The mixtures were incubated for 1 h, and centrifuged at 1200 rpm x 10 min. 0.15 ml of 1% methylcellulose overlay were added. All plates were placed at 37 °C and incubated for 3 days. On day 3, plates were stained following procedures as aforementioned in the previous sections. The modified 96 well assay was used to measure neutralizing activities of two well-characterized human anti RSV F monoclonal antibodies, Synagis® (Homaira et al., 2014), which are commercially available and D25, which was recombinantly generated in house based on the sequences derived from literature (McLellan et al., 2013). Neutralizing potency was calculated using log 10 transformed serial diluted antibody concentrations as x values, in which the cells and virus volume were not taken into account for calculation; and % neutralization as y values. EC50 values were calculated based on nonlinear fitting four parameter algorithm by GraphPadPrism (GraphPad Software).

3. Results

3.1. Establishment of a higher throughput 96 well plaque assay

To establish the 96 well plaque assay, multiple parameters were tested including initial viral inoculum, plate washing methods, infection time, HEp-2 host cell concentration, medium evaluation, detection antibody screening, infection methods, and the addition of a centrifugation step (Fig. 1). Our efforts to miniaturize the procedures and increase the throughput of the RSV plaque assay resulted in the following observations:

- It was feasible to replace manual plate washing with a plate washer, substantially increasing the throughput of the assay. The plaque counts were similar following manual versus automated plate washing. The results were consistent with different virus inoculum inputs (Fig. 1A).
- The virus infection time was reduced from 5 days to 3 days. On day 2, foci-like plaques were observed, but lytic holes in the cell monolayer were not yet formed (Supplement Fig. 1), while on day 4, plaques start to merge, resulting in ambiguous plaque counts

(Fig. 1B). On day 3, the optimal number of plaques per well that was desirable was 100 or less plaques.
- Our initial host cell concentration evaluation defined a cell input ranging from 6 to 8×10^5 cells per ml for later Design of Experiment (DoE) studies to finalize the assay conditions (Fig. 1B).
- Eliminating the host cell overnight pre-seeding step was a fundamental change from the traditional assay. Incubation of cells and virus at the same assay day achieved same results as host cell pre-seeding condition (Fig. 1B, C).
- Medium evaluation showed optimal results with EMEM medium. (Fig. 1D).
- Adding a centrifugation step, after viral infection of the cells, enhanced the infection rate, which increased the assay sensitivity (Fig. 1E).
- Removal of medium prior to addition of the overlay was not necessary (Fig. 1F), therefore enabling us to eliminate this step and simplify the assay format.
- Clear plaque visualization was achieved by immuno-staining (Fig. 1G). Both a commercially available rabbit anti-F monoclonal antibody and a cocktail of anti-F, and anti-N mouse monoclonal antibodies made in-house were equally effective for revealing viral plaques. We chose in house cocktail antibodies for the assay mainly for cost effective purpose.
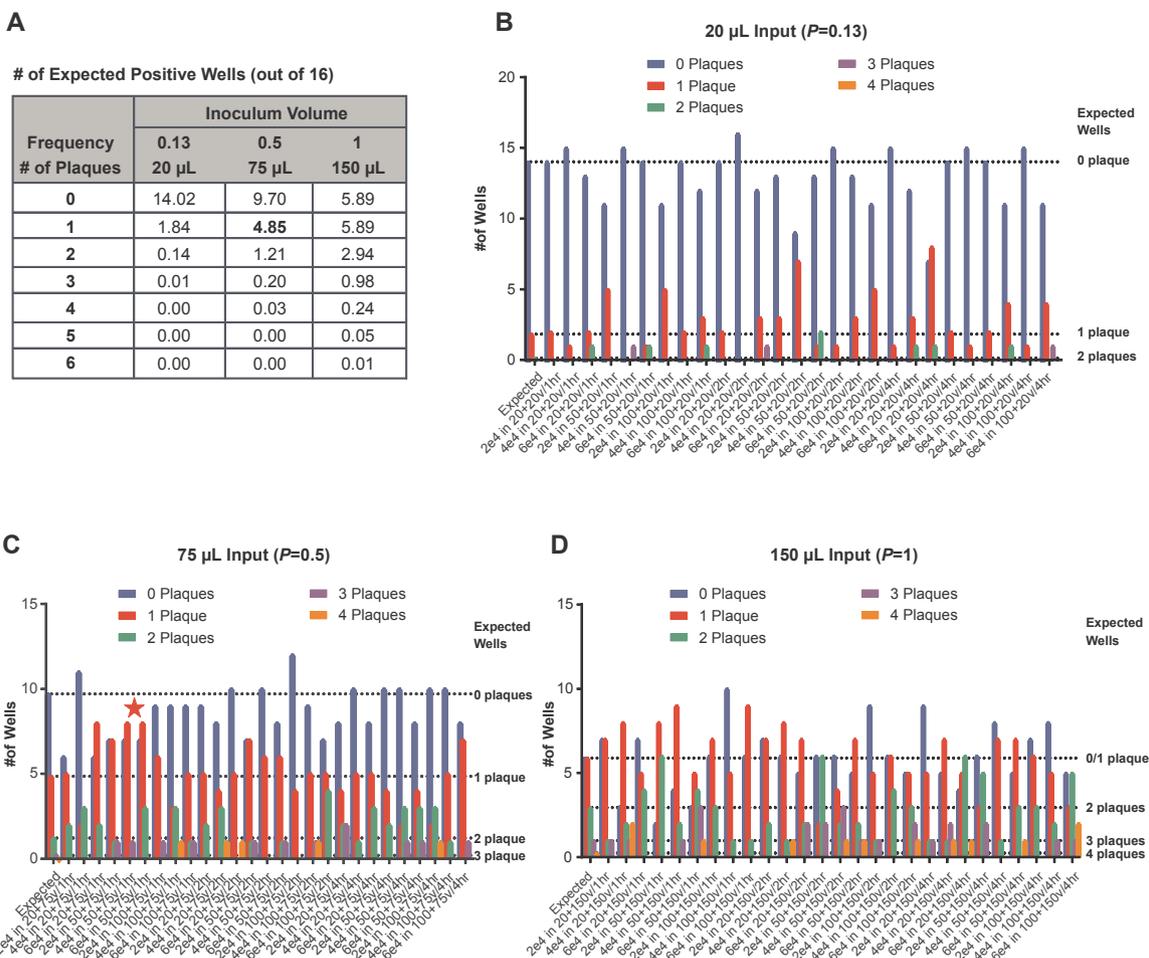
From the initial data described above, two changes in the assay protocol, elimination of pre-seeding of host cells and centrifugation of the virus/cell mixture, substantially reduced the assay time and enhanced the sensitivity of the higher throughput 96 well format assay comparing with the traditional plaque assay. To evaluate the robustness of these two observations, we ran the 96 well assays with n = 24 replicates at three different levels of input virus that targeted viral plaque forming unit at 20, 8 or 4 pfu/well, either with or without pre-seeding of cells or with and without the centrifugation step (Fig. 2). The results showed statistically significant increase in infection rate following non pre-seeding (Fig. 2A) or centrifugation conditions (Fig. 2B). The P-values were calculated by paired *t*-test method from 24 samples to compare those two conditions (Fig. 2C).

### 3.2. Deign of experiments (DoE) to finalize assay conditions

DoE was designed and performed to meet or exceed assay sensitivity of the traditional 24 well plaque assay. Four parameters were refined within one experiment: host cell density, host cell volume, viral inoculum volume, host cell and viral mixture incubation time. We evaluated the following conditions: host cell density at 2E4, 4E4 and 6E4 per well; cell volume at 20 μl, 50 μl and 100 μl per well; virus and host cell incubation time at 1, 2 and 4 h; and virus inoculum at 20 μl, 75 μl and 150 μl per well using a virus concentration of 6.7 pfu/ml. 6.7 pfu/ml is the virus input concentration to meet the limit of detection for the 24 well plaque assay. Fig. 3A showed the number of expected positive wells out of a total of 16 wells based on Poisson distributions frequency. Fig. 3B, 3C and 3D showed that the experimental results matched well with the Poisson distribution expectations for the conditions we set up. Our data indicated that the probability of obtaining positive wells containing one plaque was low with a 20 μl viral inoculum; increasing viral volume input to 75 μl or 150 μl was able to increase the probability of obtaining positive wells. The most optimal assay conditions selected were: host cells input density at a range of 4E4 to 6E4 per well; cell volume of 50 μl and virus inoculum volume of 75 μl with a virus concentration at 6.7 pfu/ml; and incubation of host cells with virus for at least 1 h followed by 10 min centrifugation at 300 x g, then added overlay for 3 days infection. This condition has been showed in Fig. 3C and marked by a red star. The final assay protocol was described in detail in the Material and Methods section 2.3.

A

Non pre-seeding vs Cell Pre-seeding
n=24

- 20 pfu/well
- 8 pfu/well
- 4 pfu/well
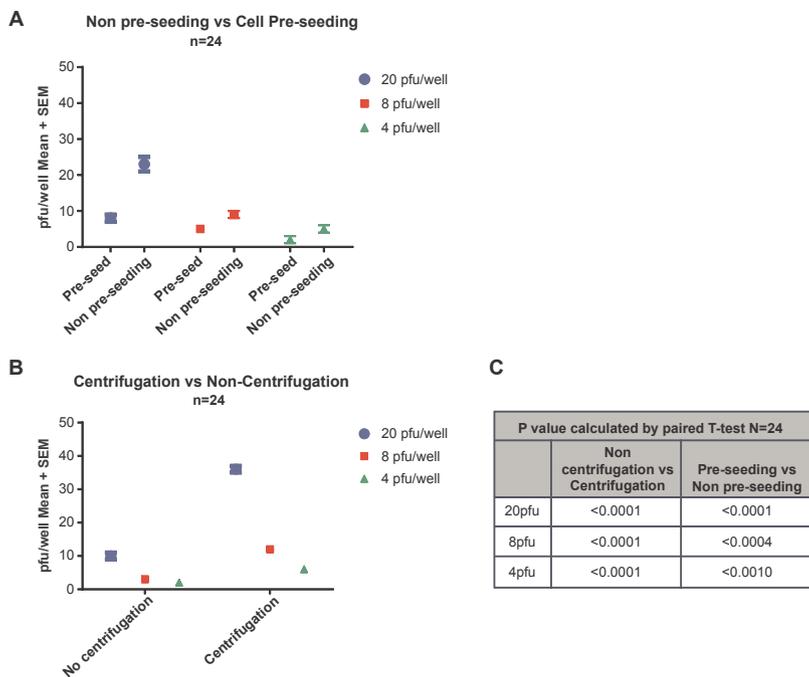
Fig. 2. Optimization of a 96 well RSV Plaque Assay. N = 24 samples were evaluated for pre-sseeding (A) and centrifugation (B). Each dot represents the mean of 24 independent wells that targeted viral plaque forming unit at 20, 8 or 4 pfu/well for HEp-2 cell host cell seeding concentration at 6×10^4 /well by pipetting 75 ul of virus and 50 ul of cells. The error bars indicate the standard error of the means. (C) P-value calculated by paired *t*-test method from 24 samples to compare those two conditions.

B

Centrifugation vs Non-Centrifugation
n=24

- 20 pfu/well
- 8 pfu/well
- 4 pfu/well

C

P value calculated by paired T-test N=24		
	Non centrifugation vs Centrifugation	Pre-seeding vs Non pre-seeding
20pfu	<0.0001	<0.0001
8pfu	<0.0001	<0.0004
4pfu	<0.0001	<0.0010

A

# of Expected Positive Wells (out of 16)

Frequency # of Plaques	Inoculum Volume		
	0.13 20 µL	0.5 75 µL	1 150 µL
0	14.02	9.70	5.89
1	1.84	**4.85**	5.89
2	0.14	1.21	2.94
3	0.01	0.20	0.98
4	0.00	0.03	0.24
5	0.00	0.00	0.05
6	0.00	0.00	0.01

B

20 µL Input (*P*=0.13)

- 0 Plaques
- 1 Plaque
- 2 Plaques
- 3 Plaques
- 4 Plaques

C

75 µL Input (*P*=0.5)

- 0 Plaques
- 1 Plaque
- 2 Plaques
- 3 Plaques
- 4 Plaques

D

150 µL Input (*P*=1)

- 0 Plaques
- 1 Plaque
- 2 Plaques
- 3 Plaques
- 4 Plaques

Fig. 3. Robust assay condition derived from DoE implementation. (A) Numbers of expected positive wells out of 16 wells, based on Poisson distributions frequency. The frequency values 0.13, 0.5 and 1 reflect the probability estimates. (B–D) Numbers of positive wells obtained from each experimental condition indicated in the x-axis, with different viral inoculum volumes. The red star represents the condition that has been selected to move forward for the finalized assay and described in material and method section 2.3 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

A

Bright Field Image

Fluorescent Image
Alexa-488 labeled anti-RSV mAb

B
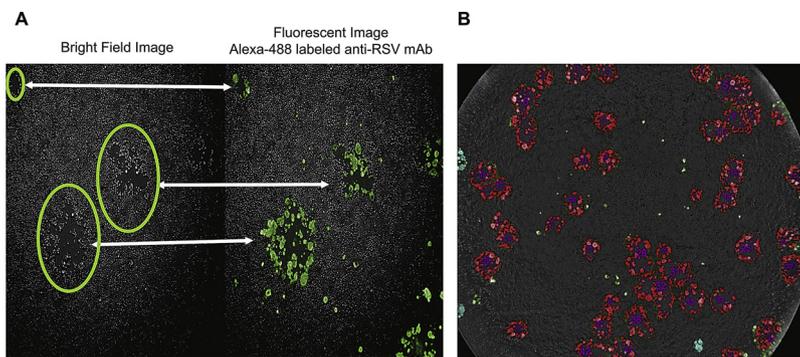
Fig. 4. Generation of an automated counting algorithm. (A) Example of combined bright field image and fluorescence image contributing to the plaque counts. (B) Result from analysis of plaques by the algorithm. Plaque lytic centers are shown in purple. The corresponding fluorescence stained plaques are shown in red. Non-Lytic plaques are shown in cyan. The algorithm counts both lytic (red) and non-lytic (cyan) plaques. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.3. Generation of the counting algorithm

PerkinElmer (PE) specifically designed an algorithm based on our requirements to count the plaques from the immuno-stained images. Both bright field image and fluorescent image acquisition capacities are required for generation of the counting algorithm. The bright field channel captures the lytic hole of the viral infection while the fluorescent channel captures the specific antibody detected viral infection based on fluorescent intensity (Fig. 4A). The combination of both the bright field and fluorescence images is required for each plaque to be included in the counting algorithm. This unique feature ensures the specificity of plaque counting and reduces background counting caused by artificial factors such as holes generated by less optimal confluent cell monolayers, "hair" like components from the culture medium that could interfere with the counting, or non-specific counts generated from background staining caused by tissue debris that are sometimes present in tissue samples. Young plaques that have not yet formed lytic holes are determined by the intensity of fluorescence, the texture thresholds and size of the plaques. These objects are added to the algorithm defined lytic plaque counts to obtain the total counts. The algorithm could count 50 or more individual plaques (up to ~100) per well of a 96 well plate, which is more than that can be detected by manual counting. Fig. 4B is an example of automated counting for RSV strains A (Long) in a single well with around 50 plaques, which is difficult to be counted manually.

We compared plaque counting with an EnSight PE algorithm to manual counting of RSV plaques by three individual analysts. Total of 1920 wells were counted by each analyst to verify the accuracy of the automated counting algorithm (Fig. 5). The result showed a high correlation between these two counting methods with R square values of 0.8 to 0.9. Higher agreement was obtained between the two methods when the plaque counts were less than 30, because it is more difficult to obtain an accurate count number manually with a high plaque number.

3.4. Applications of the 96 well plaque assay

We applied the 96 well plaque assays to quantitate infectious virus in 12 RSV virus stocks as well as nose and lung homogenates obtained from cotton rats following an experimental RSV challenge. The modified 96 well assay was also used to measure neutralizing activities of two well-characterized human anti-RSV F monoclonal antibodies, Synagis® (Homaira et al., 2014) and D25 (McLellan et al., 2013).

Case study 1: RSV virus titration was performed using the 96 well or the traditional 24 well plaque assay (Fig. 6). We tested 12 different RSV virus lots in these two assay platforms. The results indicated that virus titers obtained from the 96 well plaque assay were comparable to the titers obtained from the traditional 24 well method (R square of 0.9). Fig. 6B showed the virus titer values and Fig. 6A showed the correlation of analysis. This result confirmed the strong correlation between titers obtained by the two different plaque assay formats.

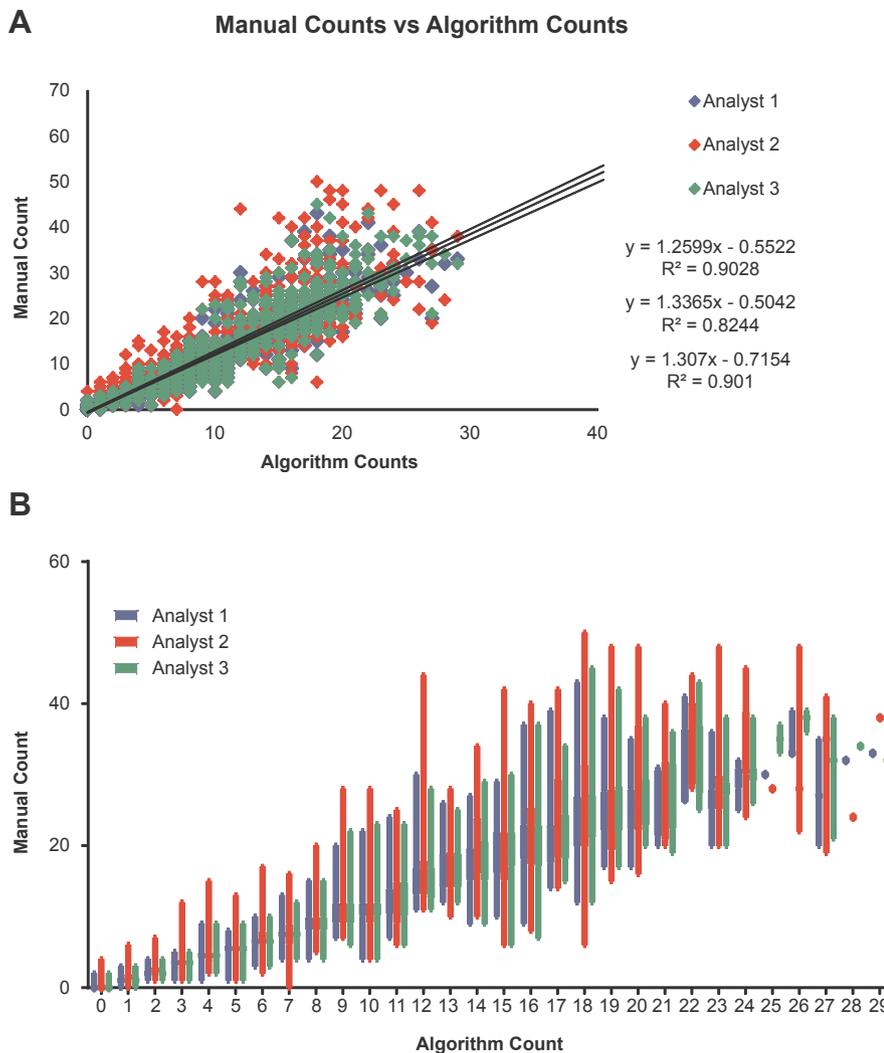Case study 2: Efficacy of RSV vaccine candidates in a cotton rat model (Fig. 7). The animal study protocol was graphed in Fig. 7A. Briefly, DS-Cav1 protein, a genetically modified recombinant RSV F protein that is able to induce potent neutralizing antibody responses in preclinical animal species (McLell and Peter, 2013), at a dose of 0.1 mcg, 1 mcg or 10 mcg with aluminium phosphate adjuvants was intramuscularly injected on D0 and D28, while WT RSV A2 virus was intranasally inoculated on D0 as a positive control. Immunized cotton rats, as well as a group of naïve animals, were intranasally challenged with RSV A2 strain (10ˆ5.5 pfu) on D56. Lung and nose tissues were harvested and homogenized on D60, 4 days post challenge. Homogenized samples were subjected to the 96 well plaque assay to determine the viral titers. The viral titers of lung and nose samples showed in Fig. 7B, with each dot representing an individual animal and a total of six animals per group. The results showed that DS-Cav1 was able to reduce the virus load in both lung and nose of cotton rats. For a dosage at 10 mcg of DS-Cav1, the efficacy was comparable to the positive control group that was intranasally treated with RSV A2. The naïve group, as the negative control, was not protected as expected.

Case study 3: Plaque reduction neutralization assay is a **gold** standard method for functional neutralizing activity evaluation, but the tedious manual counting hampers the application. We utilized this new 96 well format and further developed a plaque reduction neutralization assay to screen antibody and vaccine candidates. Fig. 8 showed an example of human RSV monoclonal antibodies targeting different RSV F epitopes. Neutralization curves of RSV mAbs D25 and Synagis® against RSV strain A2 were shown and the EC50 values agreed with published data (Zhu et al., 2017).

4. Discussion

Compared to the original 24 well plate format, a number of notable improvements have been made to this newly developed 96 well plaque assay. These include a simplified procedure, faster turnaround time, reduced requirement for host cells and reagents, and increased sample size capability. Immunostaining to identify cells expressing viral antigens was used to avoid the ambiguous "plaque" recognition by naked eye or microscopically, resulting in reduced errors caused by analysts. In conjunction with automated plate washing steps, image acquisition and algorithm counting system, this assay can be performed in a higher throughput and automated manner.

Implementation of eliminating host cell pre-seeding condition simplified the assay. In the traditional assay, seeding of host cells is normally performed one day before the assay, in consideration of recovery of host cell receptors from the possible damage of trypsin digestion. We found that for RSV, there was no difference between the pre-seeding method and non-pre-seeding method in terms of infection rate and plaque forming counts. This suggested that there was no apparent decrease in virus entry in cells exposed to RSV immediately following trypsinization. Therefore, we mixed the host cells with the virus directly on the same day of the assay instead of pre-seeding the host cells one day before the assay. This modification not only simplified the assay

A

Manual Counts vs Algorithm Counts

$y = 1.2599x - 0.5522$
$R^2 = 0.9028$

$y = 1.3365x - 0.5042$
$R^2 = 0.8244$

$y = 1.307x - 0.7154$
$R^2 = 0.901$

B

Fig. 5. Reliability of automated counting algorithm. Each of three analysts counted N = 1920 wells of plaques from optimizing viral infection time experiments that described in section 2.4 (Fig. 1B) to verify the accuracy of the automated counting algorithm. The top panel (A) shows correlation analysis results between the manual and automated counting methods based on calculated R square values for each analyst. The bottom panel (B) shows column representations of counts by the two methods from the same data set in top panel graph. The comparison is limited to counting plaques in wells with fewer than 30 plaques per well, as higher counts are difficult to manage manually.
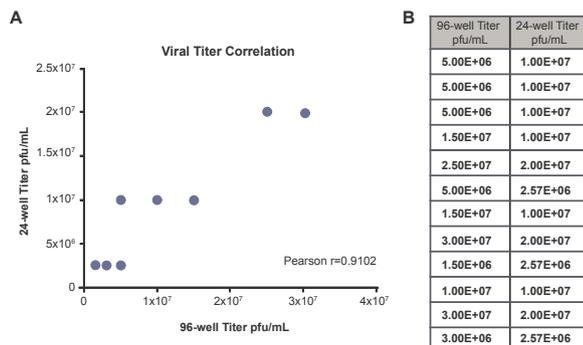
Fig. 6. RSV virus titration. 12 lots of RSV strains A or B were assessed by the 96 well format plaque assay and the traditional 24 well plaque assay. (A) Correlation between the manual and automated counting methods; (B) Titer values used for the correlation analysis in A. RSV strain subtypes and the strain names are listed.
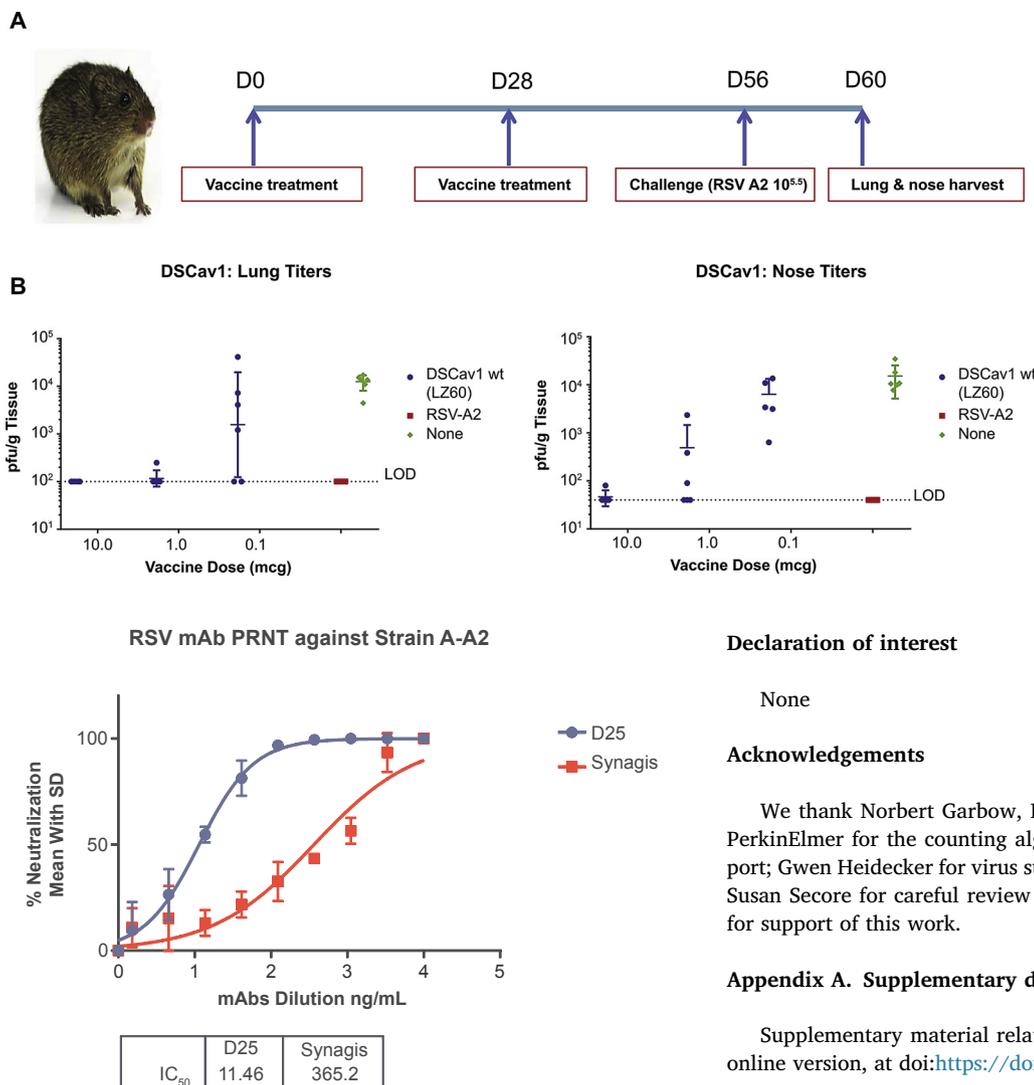
procedure, but also shortened the assay time.

It has been reported that centrifugation of RSV viruses at 700–1000 g for 60 min was able to increase detection sensitivities 2–40 fold depending on the different strains (John H.Hughes, 1993). We reported here the addition of a centrifugation step to the plaque assay improved plaque forming compared to non-centrifugation condition, making the detection sensitivity equal to the 24 well plaque assays. We hypothesized that better cell monolayer settlement might be achieved with low speed centrifugation, resulting in better infection rate.

Automated plaque imaging and counting algorithm are key factors that made this higher throughput platform suitable for rapid determination of virus concentrations for a large number of samples with higher precision and less effort. The uniqueness of the counting algorithm is its ability to distinguish lytic versus non-lytic plaques. In this report, we showed cases of successful applications of this assay for RSV which forms lytic plaque and was not effectively captured by other imagers like ELISPOT (data not shown). By adjusting the algorithm parameters, we also expanded applications of this platform to analyze large amount of samples for other respiratory virus, such as human metapneumovirus and parainfluenza virus, which form non-lytic plaques.

We compared the automated and manual plaque counts by three different analysts in Fig. 5. The R square values at ∼0.9 demonstrated that two analysts (#1 and #3) interpreted plaques similarly as the automated algorithm. The R square value at ∼0.8 indicated that one analyst (#2) might have slightly overestimated the number of plaques. This data exemplified that manual counting was prone to larger variations because of difference in the analysts' virus plaque recognition experience. In contrast, the automated approach evaluated plaque characteristics in a defined standard method that was less susceptible to variations from experiment to experiment.

In summary, we have developed a 96-well plaque assay with automated analysis and this assay greatly increased throughput and reduced labor intensity comparing to the traditional manual assay.

A

D0   D28   D56   D60

| Vaccine treatment | Vaccine treatment | Challenge (RSV A2 10^5.5) | Lung & nose harvest |

B

DSCav1: Lung Titers

DSCav1: Nose Titers

RSV mAb PRNT against Strain A-A2

	D25	Synagis
IC_{50}	11.46	365.2

Fig. 8. Plaque reduction neutralization assay. Neutralization curves of RSV mAbs D25 and Synagis® against RSV strain A (Long) are shown. D25 and Synagis® were used at a starting concentration of 10 ug/ml, and ten of 3-fold serial dilutions were applied. EC50 values were calculated based on log10 transformed antibody concentrations as x values and % neutralization as Y values with nonlinear fit (four parameters) algorithm. The error bars indicate standard deviations from duplicate samples.

5. Conclusions

We have successfully developed a higher throughput method to determine infectious RSV viral titers that allow simultaneous titration of a large number of samples in a shorter time. The automated plaque counting system has eliminated bias from identification of plaques by analysts, and proved to produce more precise and reliable titers than the manual counting method. Importantly, this platform has been utilized for functional assays, such as a RSV plaque reduction neutralization assay. This higher throughput automatic counting assay platform will greatly benefit drug/vaccine candidate screening, for which the ease and time requirement of the virus quantification is critical.

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