







## Protocol

# Rapid quantification and neutralization assays for novel coronavirus SARS-CoV-2 using Avicel<sup>®</sup> RC-591 semi-solid overlay

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**Abstract:** When working with the novel coronavirus SARS-CoV-2 during a pandemic response, having a rapid, reproducible and reliable assay for infectious virus quantitation and utilization for evaluation of potential therapeutics is critical. Compared to traditional agarose overlay plaques visualized with neutral red, assays performed with Avicel<sup>®</sup> RC-591 semi-solid overlay provide a simplified format for rapid and easy detection and neutralization testing. The method is easily modified for higher throughput using dispensers or automated processing. Fixation using formalin provides flexibility when dealing with pathogenic agents such as SARS-CoV-2 where tissue culture plates might be removed from biocontainment for staining. Although plaque assays are considered straightforward in principle, having an easily reproducible, consistent plaque assay is an invaluable tool.

**Keywords:** plaque assay; neutralization; SARS; SARS-CoV-2; coronavirus; Avicel; methylcellulose; COVID

## 1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) belongs to the family *Coronaviridae* and is the cause of the unprecedented and ongoing coronavirus disease 2019 (COVID-19) pandemic, which to date has infected more than 4 million people and caused over 300,000 deaths worldwide.[1] There is an urgent need for reliable, cost-effective and rapid assays to screen antibodies and other therapeutic compounds for neutralization activity against SARS-CoV-2.

Recently, a number of neutralization assays for SARS-CoV-2 have been reported using pseudotyped viruses[2,3] which are easier to manipulate at lower biosafety levels and allow the interrogation of specific receptor/Spike protein interactions by researchers. However, as they are specific clones these do not account for diversity of the virus that is seen in an authentic virus. Additionally, these systems do not account for interactions with any viral protein other than Spike or any step other than blocking of receptor binding, thus limiting their use. Another method for examining neutralization of serum or therapeutic compounds is a microneutralization assay based on 50% cell culture infectious dose (TCID<sub>50</sub>) endpoint dilution assay. This uses live SARS-CoV-2 and relies on the cytopathic effect (CPE) caused by the virus.[4] However, this assay takes 3 to 4 days so more rapid alternatives are sought.

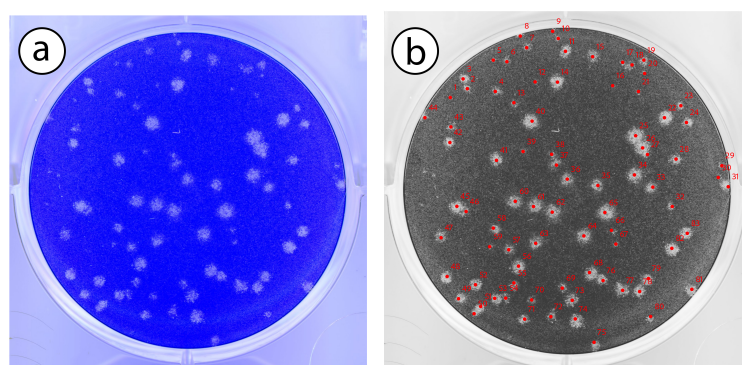
The plaque assay is the classic method for detection and quantitation of cytopathic or lytic viruses, frequently considered a gold standard for determining the concentration of live virus in a sample. Cells are grown in a monolayer and infected at various dilutions of virus, followed by an overlay. The solid or semi-solid overlay limits the viral spread to a localized region of the monolayer and following infection, this localized cell death will result in a plaque visualized after staining with crystal

violet or neutral red as a relatively clear area. There are several disadvantages to traditional solid agarose overlays that many researchers encounter, mainly surrounding the requirement to maintain the agarose at an appropriate temperature above melting that is warm enough not to solidify and clump, but not so warm as to cause damage to the cell monolayer. Clumps or bubbles in the overlay can obfuscate plaques, as will flakes or precipitation of the neutral red if used for staining. Often agarose-overlaid plates are not fixed, forcing the user into a timeline for reading and counting the plaques that may be inconvenient and not as easily suited for high-throughput or to have a second analyst verifying counts.

Many of these difficulties are overcome by use of a semi-solid overlay. Methylcellulose overlays have many advantages, but have a high viscosity making them more difficult to prepare and handle. The use of Avicel® overlays as an alternative to methylcellulose for viral plaque assays was previously described for influenza virus[5] and the coronavirus NL63[6], as well as for hemorrhagic fever viruses in biocontainment settings, including filoviruses (Ebola virus and Marburg virus[7–9]), arenaviruses and paramyxoviruses (Nipah virus[10]). Avicel® RC-591 is a dispersible co-processed blend of microcrystalline methylcellulose and sodium carboxymethylcellulose used for colloidal formulations with pharmaceutical and cosmetic applications, including lotions, creams, sunscreen, nasal sprays, and oral drug suspensions.[11] It has an extended shelf-life and is stable at ambient temperatures following autoclave sterilization procedures. In this paper we have detailed the use of Avicel® RC-591 as a low-viscosity, semi-solid overlay for plaque assays for SARS-CoV-2 detection, quantitation and plaque reduction neutralization assay.

## 2. Results

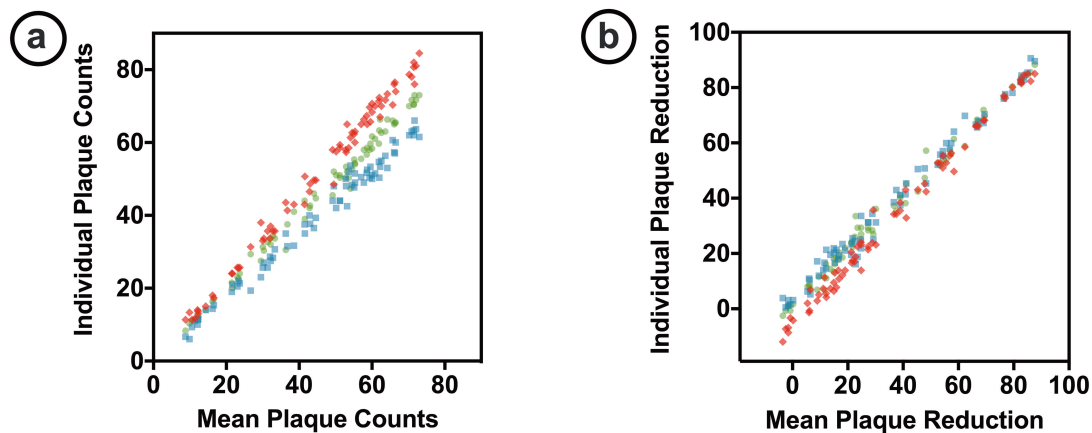
A series of plaque assays were performed in order to establish the conditions for plaquing SARS-CoV-2 using the Avicel® RC-591 semi-solid overlay. Confluent monolayers of VeroE6 cells were used in 6-well plates at one-day post-seeding, and assays were performed in triplicate using 200µL inoculum volumes and 2mL of 1X overlay without removing the inoculum. Fixing and staining two-days post-infection produced distinct, clearly countable plaques (Figure 1a). Plaques were not easily distinguishable by eye at one-day post-infection (data not shown). For ease of counting and traceability purposes, plates were scanned and counted using Adobe Photoshop, as shown in the example in Figure 1b.



**Figure 1.** Representative well from SARS-CoV-2 Avicel® RC-591 plaque assay. (a) Following crystal violet staining, plaques can be visualized and enumerated using a light box. (b) Scans in greyscale can be processed for counting using software (shown here is Adobe Photoshop v21.1.0 Count Tool).

To ensure that the assay could be performed and analyzed by multiple individuals for therapeutic screening, an experiment was conducted in which an analyst performed a plaque reduction neutralization test in triplicate and plaqued in triplicate. All of the samples from this experiment were counted by the analyst who performed the assay, as well as two independent analysts. Although there was some variability between the exact counts of the analysts (Figure 2a), each analyst trended

similarly, indicating internal consistency surrounding counting methodology. Most importantly, when the counts from each individual were used to calculate the plaque reduction and compared to the mean plaque reduction, they were similar (Figure 2b). This result demonstrates that the variation in individual counts is accounted for by the calculation of the reduction and the overall result of the experiment should not be significantly impacted.



**Figure 2.** Consistency of plaque counts between multiple analysts (a) Three analysts independently counted 72 samples and the individual counts were plotted against the mean of the three analysts. Each analyst was internally consistent with counts with low variability in counts (average %CV of 13.04). (b) When individual counts were used for calculations of plaque reduction and compared to the mean the results correlated well, indicating that effects of individual analysts should not impact the overall result of the experiment.

### 3. Discussion

Avicel<sup>®</sup> RC-591 solutions have the advantage of being simple to prepare and use, without requiring additional heating and have extended shelf-stability (similar effectiveness has been shown with other viruses up to 2 years post-preparation). The low-viscosity of the overlay and ambient stability lends towards assay optimization and automation. The assay format is easily adapted for high-throughput therapeutic screens or serum neutralization assays, with the potential to adapt to 12-or 24-well plates for higher throughput screening which would allow for use of smaller sample volumes and multiple replicates for increased statistical significance. This overlay can also be adapted to methods that use immunological detection and further optimize scale, such as immunoplaque methods that are modifiable to a 96-well format.[5]

There are gains in flexibility of assay set-up as well as with staining and enumeration, as the user has several options: stain rapidly for immediate counting, stain directly through the overlay for convenience, or fixation of the plates in 10% neutral buffered formalin for removal from containment. Because the plates are fixed and stained, plaque visibility is maintained after drying, allowing for flexibility in counting, as well as verification of counts by other analysts if enhanced data integrity is requested. This assay flexibility allows *en masse* preparation of plaque reduction assays and analysis of data over several days. In addition, establishment of an imaging system and coordination with automated counting software would further reduce analyst subjectivity, for example the Immunospot<sup>®</sup> S6 analyzer with BioSpot<sup>®</sup> software (Cellular Technology Limited, Cleveland, OH) can be used for scanning and plaque counting.

We recommend Avicel<sup>®</sup> RC-591 or other semi-solid overlays as a rapid, easy and reproducible format for plaque-based assays using SARS-CoV-2.

## 4. Materials and Methods

### 4.1. Preparation of reagents

#### 4.1.1. Media

All media are stored at 4°C for 6 months or until earliest expiration date of constituent components.

- Cell growth medium is Dulbecco's modified Eagle medium (DMEM) with GlutaMAX<sup>TM</sup> and sodium pyruvate (Gibco<sup>TM</sup>, ThermoFisher Scientific, Waltham, MA, catalog 10569) supplemented to 10% (v/v) certified US-origin heat-inactivated fetal bovine serum (HI-FBS, Gibco<sup>TM</sup>, catalog 10082).
- Plaque Assay Dilution Medium is DMEM with GlutaMAX<sup>TM</sup> and sodium pyruvate (Gibco<sup>TM</sup>, catalog 10569), supplemented to 2% (v/v) HI-FBS (Gibco<sup>TM</sup>, catalog 10082) and 1X Antibiotic-Antimycotic (Gibco<sup>TM</sup>, catalog 15240).
- For overlays, a 2X Plaque Assay Medium was prepared using 2X Modified Eagle Medium (Temin's modification, Gibco<sup>TM</sup>, catalog 11935) supplemented with 2X Antibiotic-Antimycotic (Gibco<sup>TM</sup>), 2X GlutaMAX (Gibco<sup>TM</sup>, catalog 35050) and 10% (v/v) HI-FBS (Gibco<sup>TM</sup>).

#### 4.1.2. Avicel® RC-591 2X solution for overlay

The Avicel® RC-591 microcrystalline cellulose and carboxymethylcellulose sodium used for our studies was provided by DuPont Nutrition & Biosciences (Wilmington, DE) and no additional reagents or sources were compared for these studies. However, other researchers have provided information regarding commercially available microcrystalline cellulose products and these may be useful for laboratory use.<sup>[12]</sup>

2X Avicel® RC-591 solution is prepared by adding Avicel® RC-591 to distilled water to a final concentration of 2.5% Avicel® RC-591 (w/v). For example, add 12.5g of Avicel® RC-591 per 1L bottle autoclavable bottle (glass not preferred, Corning Lexan-style bottles are ideal) and QS to 500mL with distilled water (Gibco<sup>TM</sup> 15230). Place a magnetic stir bar into the bottle and shake gently over time until homogeneous. Solution should appear as a smooth, milky liquid with no visible clumps. The solution should be autoclaved using a 15-30 minute liquid cycle and batched pooled as required in a biosafety cabinet after cool. The Avicel® RC-591 solution is stored at ambient temperature and is stable for at least 2 years from the date of preparation. Discard the Avicel® RC-591 solution if it becomes cloudy or appears contaminated.

#### 4.1.3. Staining solution

A solution of 0.2% crystal violet and 10% formalin is prepared by mixing 100mL 2% aqueous Gentian Violet (RICCA Chemical Company, Arlington, TX, catalog 3233-16) with 500mL 20% neutral buffered formalin (Richard-Allan Scientific<sup>TM</sup>, Fisher Scientific, catalog 23-751800) and QS'ing to 1L with distilled water.

### 4.2. Cells and virus

The virus stock used for these studies had been passaged three times in VERO cells and twice in VeroE6 cells and has been designated as lot NSU-V004. SARS-CoV-2 USA-WA1/2020 <sup>[13]</sup> was propagated in Vero E6 cells (BEI resources, NIAID, NIH: VERO C1008 (E6), African green monkey kidney, Working Bank # NR-596) in DMEM (Gibco<sup>TM</sup>, catalog 11960) supplemented with GlutaMAX<sup>TM</sup> (Gibco<sup>TM</sup>, catalog 35050), sodium pyruvate (Gibco<sup>TM</sup>, catalog 11360), Non-essential Amino Acids (NEAA, Gibco<sup>TM</sup>, catalog 11140), Antibiotic-Antimycotic (Gibco<sup>TM</sup>, catalog 15240) and 2% HI-FBS, Gibco<sup>TM</sup>, catalog 10082). At 66 hr post-inoculation, the supernatant was clarified by centrifugation and the concentration of HI-FBS was QS'd to 10% final concentration. All procedures performed with the virus were conducted in a Biosafety level 4 (BSL4) laboratory.

#### 4.3. Detailed plaque assay protocol

1. Plate Vero E6 cells at  $6-8 \times 10^5$  per well in 6 well plates (Corning® CellBIND catalog 3335) to achieve confluency the next day.
2. Serially dilute samples in Plaque Assay Dilution Medium.
3. Decant, aspirate or pipette growth medium from 1-2 plates at a time and add 200  $\mu$ L of diluted samples to appropriate wells in triplicate.
4. Adsorb samples to the cell monolayers for 1 h at 37°C and 5% CO<sub>2</sub> with gentle rocking approximately every 15 minutes to prevent monolayer drying. For a large number of plates, it is necessary to incubate and rock in groups.
5. Pre-warm 2X Plaque Assay Medium and 2X Avicel® RC-591 solution to 37°C in a waterbath or an electronic bead bath. Prepare the overlay by combining Avicel® RC-591 2X Overlay and 2X Plaque Assay Media 1:1 to obtain a 1X concentration and mixing well by inversion. The 1X mixture should not be stored for extended periods.
6. Without removal of the inoculum, overlay the monolayers with 2mL per well of a 1:1 mix of 2.5% Avicel® RC-591 solution mixed with 2X Plaque Assay Medium using a serological pipette or dispensing pipettor (ex. Eppendorf® Repeater®/Combitips advanced® system).
7. Swirl plates gently to mix and incubate at 37°C and 5% CO<sub>2</sub> for 2 days.
8. Gently swirl plates to loosen the Avicel® RC-591 and discard the overlay to waste. Fix plates with 10% neutral buffered formalin before removing from containment, then stain with 0.2% crystal violet staining solution for 30 minutes. Rinse under tap water and dry prior to enumeration.

#### 4.4. Detailed plaque reduction neutralization assay

1. Plate Vero E6 cells at  $6-8 \times 10^5$  per well in 6 well plates (Corning® CellBIND catalog 3335) to achieve confluency the next day.
2. Serially dilute therapeutic test article (TA) in a 96-well polypropylene deepwell plate (Nunc, catalog 278743). Polystyrene or tissue-culture treated plates are avoided as antibodies may adsorb tightly. Ensure that negative control and if possible, known positive controls are included. Example dilution series included:
  - (a) Two-fold dilutions:
    - i. Start with 650  $\mu$ L of TA at highest concentration in first well and 325  $\mu$ L of Vehicle (TA resuspension buffer, or PBS, as appropriate) in remaining wells.
    - ii. Serially dilute 325  $\mu$ L, mixing at least 5 times (gently, avoiding air bubbles) at each step.
    - iii. Change pipette tips and transfer to next well.
    - iv. Discard final 325  $\mu$ L. 325  $\mu$ L should remain in each well.
  - (b) Log dilutions:
    - i. Start with 360  $\mu$ L of TA in first well and 324  $\mu$ L of Vehicle in remaining wells
    - ii. Serially dilute 36  $\mu$ L, mixing at least 5 times (gently, avoiding air bubbles) at each step.
    - iii. Change pipette tips and transfer to next well.
    - iv. Discard final 36  $\mu$ L. 324  $\mu$ L should remain in each well.
  - (c) Half-log dilutions:
    - i. Start with 475  $\mu$ L of TA in first well and 325  $\mu$ L of Vehicle in remaining wells.
    - ii. Serially dilute 150  $\mu$ L, mixing at least 5 times (gently, avoiding air bubbles) at each step.
    - iii. Change pipette tips and transfer to next well.
    - iv. Discard final 150  $\mu$ L. 325  $\mu$ L should remain in each well.
  - (d) Quarter-log dilutions:
    - i. Start with 800  $\mu$ L of TA in first well and 350  $\mu$ L of Vehicle in remaining wells.
    - ii. Serially dilute 450  $\mu$ L, mixing at least 5 times (gently, avoiding air bubbles) at each step.
    - iii. Change pipette tips and transfer to next well.

- iv. Discard final 450 $\mu$ L. 350 $\mu$ L should remain in each well.
3. Seal plates with sterile, breathable plate sealer for transfer to biocontainment (CELLTREAT scientific products, catalog 229130).
4. Dilute virus in DMEM Plaque assay diluent to a concentration of 100 PFU per well for 6 well plate (1x10<sup>3</sup> PFU/mL, as 200 $\mu$ L is used for assay) sufficient for number of samples.
5. Using a multichannel pipette, mix diluted virus 1:1 with diluted TA in the deepwell plate (either 325 $\mu$ L or 350 $\mu$ L added per well, depending on dilution series). For calculation purposes, at least one set of samples were included that were diluent + virus only.
6. Seal deepwell plates with a fresh sterile, breathable plate sealer and incubate at 37°C for 60  $\pm$  10 minutes.
7. Following this incubation, remove all media from the 6-well plates by pipetting, aspiration or decanting and add 200 $\mu$ L of each dilution to the appropriate wells in triplicate. Mock-infected wells are included as negative control.
8. Incubate plates at 37  $\pm$  4°C, 5  $\pm$  1% CO<sub>2</sub> for 60  $\pm$  10 minutes, rocking gently approximately every 10  $\pm$  5 minutes, and overlay with 2mL of 1:1 mixture of Avicel<sup>®</sup> RC-591 2X Overlay and 2X Plaque Assay Media as in plaque assay method.
9. Following 2 day incubation at 37°C and 5% CO<sub>2</sub>, plates are fixed, removed from biocontainment, stained and counted.
10. To determine the IC<sub>50</sub> values in GraphPad Prism v.8.4.2 software, from an XY data table of percent reduction on the Y axis (using the diluent + virus-only control set to 100%) versus the logarithm of the concentration of the inhibitor (X axis) use the nonlinear regression analysis, "Dose-response curves – Inhibition" "log(inhibitor) vs. response." The IC<sub>50</sub> is the concentration of agonist that gives a response half way between Bottom and Top (where Top and Bottom are plateaus in the units of the Y axis). This is not necessarily the same as the response at Y=50, and the values of Bottom and Top, the IC<sub>50</sub> may give a response nowhere near "50". Prism reports both the IC<sub>50</sub> and its log.

*Note: Final compound concentrations in assay wells are twofold less than that in the compound dilution plate due to the addition of virus during incubation step.*

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**Conflicts of Interest:** The authors declare no conflict of interest.

#### **Abbreviations**

The following abbreviations are used in this manuscript:

BSL	Biosafety level
CPE	cytopathic effect
DMEM	Dulbecco's modified Eagle medium
FBS	fetal bovine serum
HI	heat-inactivated
IC <sub>50</sub>	50% inhibitory concentration
PFU	plaque-forming unit
TCID <sub>50</sub>	50% cell culture infectious dose
TA	test article
QS	<i>quantum satis</i>

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