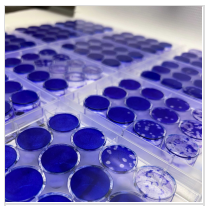

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Influenza virus plaque assay

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Steven F Baker: Adapted from A. Mehle, University of Wisconsin-Madison

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Determine virus concentration from cell culture, lung homogenates, and more. This protocol measures virus in plaque forming units per ml solution.

This protocol differs from classic flu plaque assays by using a semi-solid overlay (Avicel) as opposed to agarose. This is beneficial in avoiding reproducibility issues that can arise from burning monolayers with hot overlay, and is faster to make and aspirate. The negative is that you cannot plaque purify viral isolates as you can with agarose.

Theoretically one infectious virion can spawn one plaque by initiating in a cell and infecting neighboring cells. This is the gold standard for virology, and for influenza, the plaque:particle ration is ~ 1:100. Thus there are lots of non/semi-infectious or defective interfering particles that are produced that are not on their own infectious.

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plaque, concentration, pfu, titer, semi-solid overlay

protocol ,

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This protocol uses 12-well plates. Best practice is to use technical triplicates (or duplicates at the minimum). Serial 10-fold dilutions across the plate commonly account for 4-logs (horizontal) or 3-log (vertical) resolution.

- MDCK cells
- D10 - DMEM **Corning Catalog #10-013-CV** + 10%

  FBS **Peak Serum,**

  Inc Catalog #PS-FB3

  Trypsin
- [0.25%] **Catalog #25-053-CI**
- VGM (***recipe follows***)
- 2X DMEM (***recipe follows***)
- 2.4% Avicel (***recipe follows***)

  TPCK-trypsin **Millipore**
- **Sigma Catalog #T1426-50MG** - 2 mg/ml in DMEM, sterile-filtered. Aliquots stored at -80 C
- DPBS **Corning Catalog #21-031-CV** , sterile
- DPBS, non-sterile
- 70% Ethanol
- Crystal violet (***recipe follows***)
- 12-well tissue-culture treated plates, tissue culture-treated

VGM (Virus Growth Medium)

To make 500 ml

- 500 ml DMEM **Corning Catalog #10-013-CV**

  BSA for tissue
- 12.5 ml 7.5% culture **Catalog #A2058-100G** in DPBS, sterile-filtered

  HEPES [1
- 12.5 ml M] **Catalog #25-060-CI**

  Pen/Strep
- 5 ml [100X] **Catalog #30-002-CI**

- Add additives to media bottle in hood, keep sterile, store at 4 °C

2X DMEM

To make 1 L

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☒DMEM

- 26.8 g powder **Corning Catalog #50-013-PB**
- milli-Q H2O
- 7.4 g sodium bicarbonate
- 1 N HCl (or 1 N NaOH)
- Vacuum filter sterilization units (4, 250 ml ea, 200 um)

☒BSA for tissue

- 50 ml culture **Catalog #A2058-100G** 7.5% in DPBS, sterile-filtered

☒1 M

- 50 ml HEPES **Corning Catalog #25-060-CI** , sterile

☒200 mM

- 20 ml Glutagro **Corning Catalog #25-015-CI** , sterile

☒100X

- 10 ml Pen/Strep **Corning Catalog #30-002-CI** , sterile

- In 1 L TC graduated cylinder, add ~790 ml milli-Q H2O and TC stir bar, stir
- Slowly add 26.8 g DMEM powder
- Stir 30 min
- Add 7.4 g sodium bicarbonate ($NaHCO_3$)
- Fully dissolve
- Calibrate the pH meter with standards
- Adjust solution to pH 7.0 with 1 M HCl (or 1 N NaOH)
- Bring to final volume of 870 ml exactly
- Stir 30 min
- Filter sterilize 217.5 ml into four (0.2 µM) 250 ml stericup bottles
- Move the filtered solution into BSC to switch filter for caps, ethanol-ing into the hood
- In the BSC to each 250 ml unit, add 12.5 ml HEPES, 12.5 ml TC BSA, 2.5 ml pen/strep, 5 ml glutagro
- Store at 4 C

2.4% Avicel

To make 150 ml in a 250 ml TC bottle

- 150 ml milli-Q H2O
- 1 stir bar
- 3.6 g ☒Avicel **Dupont Catalog #RC-581** – very fluffy, be careful

- Stir until evenly resuspended, this takes a while
- Autoclave (liquid cycle, 30 min)
- Stir again
- Store @ room temperature

0.3% Crystal violet

To make 500 ml

Start in the morning, wear gloves & lab coat, and put down bench paper

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Crystal

- 1.8 g Violet **Amresco Catalog #0528-100G**
- milli-Q H2O

- In a 500 ml plastic bottle, add:
  an already purple stir bar
  600 ml milli-Q H2O
  1.8 g crystal violet
- Stir for a long time - consider heating to 42 C also
- Filter with Whatman #1 paper and an already purple funnel into second plastic bottle
- Store at room temperature

All manipulations should take place in biosafety cabinet wearing proper PPE

Bring DMEM, VGM, trypsin, PBS, 2X DMEM to 37 C in water bath

Seed cells (day 1) 1d

1 Collect and count MDCK cells

For the following steps, MDCK cells are maintained in 10 cm dishes in 10 ml volume of D10

1.1 Wash & collect cells

- Aspirate D10, add 4 ml sterile DPBS, aspirate
- Trypsinize cells with 1 ml 0.25% trypsin for 5-15 min at 37 C, tap gently to lift off
- Resuspend in 10 ml D10
- Count cells by adding 10 ul to counting slide and measure on TC20 or hemacytometer

1.2 Prepare enough cells to seed 12 well plates with 1 ml per well

- Resuspend cells to 3E5 cells/ml
- Add 1 ml per well in a 12 well plate
  Always fill all wells in a plate with liquid, even if all wells are not needed. This prevents evaporation effects
- Incubate cells at 37C, 5% CO_2 overnight

Prepare virus dilutions (day 2) 2h

2 Prepare virus dilutions

Enveloped viruses are sticky and can adhere to plastic pipet tips. It's crucial to change tips between each dilution step, and pipet up & down a consistent number of times. For example,

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pipet up/down once, move liquid to next dilution, pipet up/down 8 times; discard tips, repeat.

2.1 From mouse lungs

Lungs should be collected in individual Eppendorf tubes and flash frozen. To flash freeze, submerge closed tube in liquid nitrogen. Frozen samples are stored at -80 C.

On day of infection, prepare lung homogenates as follows:
- Thaw lungs on ice for >1 h
- Weigh lungs, move to bead buster 2 ml eppie tubes that each contain 1 magnetic ball bearing
- Add 0.5 ml VGM to rinse original lung tube and add to 2 ml tube. Up to 1 ml liquid volume can be used
- Lyse lungs using Qiagen Tissue Lyser II
- Program 4: 3 cycles, 30 Hz, 30 s. In between cycles, move tubes to ice for ~1 minute
- Spin homogenates 10,000 x g at 4 C for 10 min, **transfer supernatant (containing virus) to new eppie tube, continue to dilutions in step 2.2 below**
- Transfer beads to 70% ethanol to sterilize, rinse thoroughly with DI H_2O, and store for later autoclaving

2.2 From tissue culture-grown virus stock

Infection inoculums for 12-well plates use 0.1 ml virus, when preparing dilutions, be sure to have adequate volume. Prepare 10-fold serial dilution series either in 96-well V-bottom plates or eppendorf tubes.

It may feel like less work to dilute in eppendorf tubes (less dilutions!) but by using a plate, you can use a multichannel pipette to simultaneously dilute samples. Plus the "technical replicate" portion is more faithful. E.g. if I'm just quick tittering 1-2 viruses I'll opt for eppendorf tubes. Most other situations I use 96-well plates.

Prepare in 96-well V-bottom plates (use each column as a technical singlet, ie, use 3 columns for triplicate):
Add 135 ul VGM to all wells
Add 15 ul stock solution to row A
Dilute stock solution serially, transferring 15 ul stepwise
Prepare in eppendorf tubes (pull from eppendorf tubes to inoculate technical replicates):
Add 360 ul VGM to eppendorf tubes
Serially dilute 40 ul

Infect cells (day 2)

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3 Infect cells 01:00:00

1h

3.1 Inoculate

- Wash MDCK cells by aspirating, adding ~0.5 ml VGM per well
- Aspirate, add 0.1 ml virus inoculum
- Incubate at 37 C

Critical to label plate with accurate description of dilution series/virus used

3.2 Infect for 1 h at 37C

- Rock plate every ~15 min by drastically tipping forward-back and side-side. Don't allow inoculum only to sit on the well edges or monolayer to dry out

3.3 Prepare overlay

Each well will receive 1 ml overlay, determine final volume needed
- Add 2X DMEM into 50 ml falcon tubes, add TPCK-trypsin to ~1 ug/ml (volume calculated after 2X diluted with Avicel). However, you need to first mix TPCK-trypsin with 2X DMEM before adding avicel, or the mixture won't be uniform concentration
- Dilute 2X DMEM 1:1 with Avicel
- Store these 50 ml tubes in 37 C water bath until ready to stop infection

3.4 Stop infection by adding overlay

- Add 1 ml overlay per well (no need to aspirate virus inoculum!)

Critical to vigorously swirl plate after inoculum is added. The goal is to get an even mixture of inoculum/overlay. If you don't do this and maintain a liquid interface at the cell surface, plaques will be amorphous and hard to differentiate.

3.5 Incubate at 37 C for 3 days

3d

72:00:00

Some fast growing viruses like PR8 or WSN can be developed after 2 days, but 3 days is best to be safe.

Develop (day 5) 1h

4 Stop infection and visualize plaques with Crystal Violet

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4.1 Aspirate wells

- Avicel-containing overlay can be aspirated into the trap (hooray!). In BSC, tip plate towards you and aspirate overlay, trying to collect it all.

4.2 Wash

- Add ~0.5 ml PBS (does not have to be sterile) to each well. I just lightly dispense across the plate without measuring
- Swirl the liquid in the wells to dislodge remaining overlay
- Aspirate

4.3 Fix

10m

- Add ~0.5 ml 70% ethanol to each well
- Incubate for **10 minutes at room temperature**
- Aspirate

00:10:00

4.4 Stain

10m

- Add ~0.5 ml Crystal Violet to each well, making sure that liquid completely coats the surface
- Incubate for **10 minutes at room temperature**
- Aspirate into Crystal Violet trap in the Kajon lab fume hood

00:10:00

4.5 Wash

- Add tap water to fill the wells of the plate and dump down the sink
- Repeat for a total of 4 washes

4.6 Dry

- Dry plates by leaving upside down at an angle to encourage air flow
- On top of bench pad in the fume hood

Count and titer

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5 Titers are represented in plaque forming units (pfu) per ml stock
From animal infections, often represented as per g tissue. Be sure to weigh tissue so that you can back calculate later

5.1 Aim to count 10-30 plaques per well

5.2 Calculate titer by multiplying average number of plaques by reciprocal dilution factor and by inoculum volume

For example, and average of 3 plaques are observed in the 10^{-5} dilution well
3×10^{5} [10^{-5} dilution] X 10 [0.1 ml inoculum]

Save yourself some time by developing a spreadsheet template like this one

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