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SeV titration using FFU

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Protocol status: Working
We use this protocol and it's working

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Abstract

Protocol for titration of SeV

Materials

Infection medium

- DMEM ..... 500 mL
- Pen/Strep ..... 5 mL
- 35% BSA ..... 5 mL
- 5% $NaHCO_3$ ..... 12 mL

2X Infection medium

- DMEM F12 (Powder) ..... 1 sachet
- Pen/Strep ..... 10 mL
- L/Glut .....10 mL
- 35% BSA ..... 6 mL
- HEPES 1M ..... 10 mL
- $NaHCO_3$ ..... 18 mL
- Sterile dH_2O ..... to 500 mL

Overlay medium

- 2X Infection media
- 2.5% Sterile Avicel
- TPCK-Trypsin

Reagents for Staining

- Anti-SeV primary antibody (example: ms SeV NP antibody, Clone M73/2)
- Goat anti-mouse H+L Biotinylated Antibody (Southern Biotech #1036-08)
- Streptavidin AP (ThermoFisher #21324)
- 1-Step™ NBT/BCIP Substrate Solution (ThermoFisher #34042)

Preparing Overlay Medium

1 To prepare the overlay medium, first, add the correct amount of TPCK to the 2X infection medium. Then mix the 2X infection media/TPCK with the same volume of 2.5% Avicel to prepare a 1:1 solution. Alternately, 0.6% Agarose can be used in a similar 1:1 manner for a final concentration of 0.3% Agarose. All media must be prepared in sterile conditions.

Example for 50 mL of Overlay medium using Avicel:

- 25 mL of 2x infection medium
- 50 µL of TPCK (2 mg/mL)
- 25 mL of 2.5% Avicel

Day 0 - SEEDING CELLS

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- Seed 2×10^5 LLCMK-2 cells/well in a 24-well plate (or in a way they are 100% confluent next day).
  - *Suggestion:*
    - Trypsinize a whole, 80% confluent T75 flask;
    - Resuspend cells in 10 mL (regular 10% DMEM) and seed 450 µL each well - Homogenize and check if cells are evenly distributed in the wells.
    - If scaling up to 48-well plates, seed 10^5 cells/well.
- Incubate plates at 37 °C.

Day 1 - DILUTION AND INFECTION 1h

3 1h
- Check cells and ensure they are 100% confluent.
- Prepare 10fold dilutions of your samples in Infection Media;
  - Change tips for every dilution;
- Wash cells twice with PBS to remove any FBS trace;
- Inoculate 200 µL of your serially diluted samples on each well (if using 48-well plates inoculate 180 µL).
  - This step can be done in duplicates or triplicates.
  - Inoculate in the opposite direction from the dilution so there is no need for changing tips;
  - Save wells for the negative control (Infection media only) and the 2ry antibody negative control (Also infection media only).
- Incubate at 37 °C for 01:00:00, gently rock the plate every 10';

- Remove the inoculum and add 500 µL of Overlay medium + TPCK 2 µg/mL (250 µL if using 48-well plates).
- Incubate the plates for 2 days at 37 °C .

Day 3 (48hpi) - FIXATION

2h

4

- Aspirate the overlay medium from the wells and fix cells with 10% Formaldehyde (in PBS) for 02:00:00 . If using overlay medium containing Agarose, directly add 500uL 4% PFA over the wells and incubate as follows.
  - 200 µL/well
  - Use the plate rocker to avoid drying
- Remove the fixative agent and wash twice with PBS.
- Store at 4 °C or move directly to the staining steps.

2h

Day 3 - STAINING

1h

5

- Remove the PBS and replace with 150 µL of PBS/1% BSA 1%/0.1% Saponin.
- Incubate for 30' to 1hr to block/permeabilize cells

1h

OBS.: All the following steps are done with 150 to 180 µL/well of reagent. Scale up according to the number of wells. To achieve better results also keep the plates on the rocker for all the following incubation steps.

- Aspirate the blocking/permeabilization solution and incubate with primary antibody;
  - Unlabeled **Ms anti-SeV NP (1.3 mg/mL) M73/2**
  - 1:2000 diluted in PBS/1% BSA 1%/0.1% Saponin;

OBS.: Don't add the primary antibody to the 2ry-only negative control well.

- Incubate for 01:00:00 - rocking

- Wash 3x with PBS (180 µL of PBS/well, aspirate carefully for not scraping the monolayers).
- Incubate with secondary antibody for 30';
  - Biotinylated Gt anti-Mouse (H+L) diluted 1:1000 in PBS/1% BSA 1%/0.1% Saponin.
- Wash 3x with PBS
- Incubate with Streptavidin for 30'
  - AP-Conjugated Streptavidin, diluted 1:1000 in PBS/1% BSA 1%/0.1% Saponin.
- Wash 3x with PBS
- Incubate with AP substrate for up to 20' (or until the FFUs are pretty stained).

- 1-Step NBT/BCIP Substrate solution for AP

- Wash 1X with dH_2O.

Day 3 – FOCUS COUNTING

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- In the last dilution with visible focus, count the stained focus in all the wells from that particular sample and divide by the number of replicates.
- The titer will be expressed by the average number of focus counted times the dilution factor, per volume of initial inoculum.

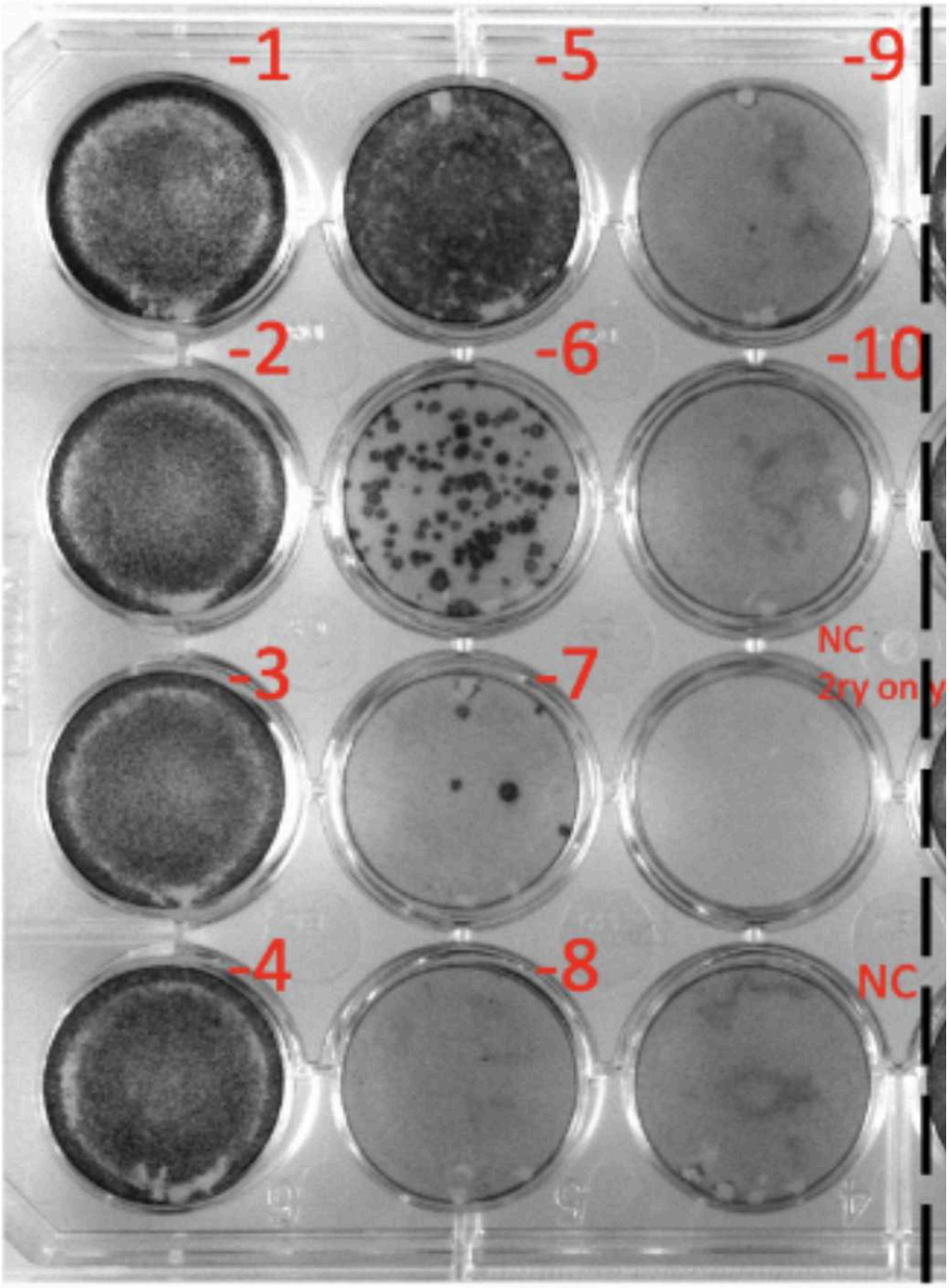

Figure 1. Representative titration plate. 10-fold dilutions of the initial virus inoculum are indicated in red. Infection media was used as negative control (NC), and is indicated in the bottom-right. Fixed NC monolayer was either incubated with the secondary (2ry) antibody only, or both primary and secondary antibodies as indicated.

- Example: In the plate above, there are 5 FFUs in the -7 dilution. Considering the dilution factor, the titer would be 5×10^7 FFUs. Finally considering the initial volume inoculated (200 uL), the final titer of that particular sample is 5×10^7 FFU/200uL.

Protocol references

Annotated by IAC