LCMV Plaque Assay on Vero Cells

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Purpose

This protocol describes a standard plaque assay for determining the infectious titer of LCMV stocks using Vero cell monolayers. The readout is plaque-forming units per milliliter (PFU/mL).

⚠ LCMV biosafety and handling

LCMV is a zoonotic arenavirus capable of causing severe disease in humans, including meningitis and congenital infection. Work with live LCMV must:

- Be conducted under the appropriate institutional **biosafety level (typically BSL-2 with additional local conditions)** and approved animal and biosafety protocols.
- Use **dedicated infectious CO incubators** and clearly labelled containment for all cultures and waste.
- Include suitable **PPE** (lab coat/gown, gloves, eye protection as appropriate) and measures to avoid sharps injuries, aerosol formation, and spills.
- Exclude or take special precautions for **pregnant or immunocompromised personnel** according to institutional policies.

All waste (liquid and solid) must be inactivated according to local regulations (e.g. chemical disinfection and/or autoclaving) **before** disposal.

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Principle

- Serial 10-fold dilutions of virus are prepared in serum-containing medium.
- Vero cell monolayers are infected with each dilution for a defined adsorption period.
- Inoculum is removed and cells are overlaid with a semi-solid medium to restrict virus spread to neighboring cells.
- After several days of incubation, individual plaques (zones of lysis/infection) are fixed, stained, and counted.
- Viral titer is calculated from plaque counts, dilution factor, and inoculum volume.

Materials

Cells and Media

- Vero cells (African green monkey kidney)
- Vero cell propagation medium (see `buf_vero_propagation_medium.qmd`)
- LCMV plaque assay medium (2× MEM-based overlay; see `buf_lcmv_plaque_assay_medium.qmd`)
- Methylcellulose or agarose for overlay (if preparing a gel-based overlay)

Virus

- LCMV stocks to be titrated (Armstrong, Clone 13, WE, Docile, etc.)
- Known reference virus stock (optional internal control)

Reagents and Consumables

- PBS without Ca^2 /Mg^2
- Crystal violet staining solution (e.g. 0.5% crystal violet in 20% ethanol)
- Fixative:
  - 4% paraformaldehyde in PBS, or
  - 10% neutral buffered formalin
- Sterile 6-well plates or 12-well plates
- Sterile conical tubes (15 mL, 50 mL)
- Micropipettes and sterile filtered tips
- Serological pipettes
- Waste containers with disinfectant (e.g. 10% bleach)

Equipment

- Class II biosafety cabinet
- CO incubator set to 37°C, 5% CO
- Inverted light microscope
- Water bath (for overlay preparation if using agarose)
- Aspirator or vacuum system with appropriate trap and disinfectant
- Laboratory timer

Preparation

Vero Cell Seeding

1. One day before infection, seed Vero cells in 6-well plates to achieve a **90–100% confluent monolayer** on the day of infection.
2. Use Vero propagation medium and incubate at 37°C, 5% CO .

> **Tip**
>
> Aim for evenly distributed, healthy monolayers. Avoid overconfluency and large areas of cell-free plastic.

Overlay Medium

You can use either:

- **Methylcellulose-based overlay**, or
- **Low-melting agarose overlay**

A common approach is to mix **equal volumes** of:

- 2× plaque assay medium (see buffer file) and
- 2% methylcellulose or 2% agarose (cooled to ~42–45°C for agarose)

to obtain a final 1× medium with 1% methylcellulose or 1% agarose.

Prepare and keep overlay medium **warm (37–42°C)** until use.

Virus Dilution Series

1. Thaw a virus aliquot rapidly in a 37°C water bath and immediately place on ice.
2. Prepare a series of 10-fold dilutions in Vero propagation medium (e.g. 10^{-1} to 10^{-}).
3. Use sterile 1.5 mL or 15 mL tubes and mix gently by pipetting.

Example:

- Tube 1: 900 µL medium + 100 µL virus stock (10^{-1})
- Tube 2: 900 µL medium + 100 µL from Tube 1 (10^{-2})
- Continue to the desired dilution (e.g. 10^{-}).

Infection of Vero Cell Monolayers

1. Remove growth medium from each well of the 6-well plate.
2. Rinse wells once with PBS (optional but recommended).
3. Add virus dilutions:
    - Typically 200–300 µL per well.
    - Use at least duplicate wells per dilution if possible.
4. Gently rock plates to distribute inoculum evenly.
5. Incubate at 37°C, 5% CO for **1 hour**.
6. Gently rock plates every 10–15 minutes during adsorption.

Overlay Application

1. After the adsorption period, carefully aspirate the virus inoculum from each well without disturbing the cell monolayer.
2. Add **2–3 mL** of warm overlay medium to each well.
3. Swirl plates gently to ensure even coverage.
4. Incubate plates at 37°C, 5% CO for **3–5 days**, depending on strain and desired plaque size.
    - Armstrong and WE often show clear plaques by day 3–4.
    - Clone 13 and Docile may require up to day 5 for optimal plaque definition.

Fixation and Staining

1. At the chosen endpoint, remove plates from the incubator.
2. Carefully add an equal volume of **fixative** (e.g. 4% paraformaldehyde) directly on top of the overlay to fix cells and inactivate virus.
    - Incubate at room temperature for **30–60 minutes**.
3. Aspirate overlay and fixative into a disinfectant-containing waste container (e.g. 10% bleach).
4. Gently rinse wells with tap water or PBS.
5. Add sufficient **crystal violet staining solution** to cover the monolayer (e.g. 1–2 mL per well).
6. Incubate at room temperature for **15–30 minutes**.
7. Rinse thoroughly with tap water until excess stain is removed.
8. Air-dry plates.

Plaques will appear as clear (unstained) zones against a darkly stained monolayer.

Plaque Counting and Titer Calculation

1. Count plaques in wells with **10–100 plaques** for accurate quantification.
2. For each counted well, record:
    - Dilution factor (e.g. 10)
    - Number of plaques
    - Inoculum volume (e.g. 0.2 mL)

3. Calculate PFU/mL:

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PFU/mL = (Number of plaques × Dilution factor) / Volume of inoculum (mL)
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Example:

- 45 plaques at a 10 dilution with 0.2 mL inoculum

PFU/mL is calculated as:

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PFU/mL = (45 × 10^5) / 0.2 = 2.25 × 10^7 PFU/mL
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4. Average PFU/mL from replicate wells at the same dilution.

Controls and Quality Checks

- Include at least one **mock-infected well** (no virus) to check for contamination and monolayer health.
- Optionally include a **reference virus stock** of known titer to verify assay consistency.
- Plaques should be well separated and have clearly defined borders.
- Monolayers should remain intact in mock-infected wells, with no evidence of cytotoxicity from medium or overlay.

Troubleshooting

Issue	Possible cause	Suggested action
No plaques at any dilution	Virus stock inactive or too dilute	Check storage history; test a less diluted stock
Too many plaques (confluent)	Dilutions not high enough	Extend dilution series (e.g. to 10 or 10)
Irregular or fuzzy plaques	Overlay too thin or too thick; incubation off	Adjust overlay volume; verify incubator conditions
Poor cell monolayer	Cells not healthy or not fully attached	Improve cell culture conditions before assay
High background staining	Incomplete washing after staining	Increase number and volume of wash steps

Safety considerations

- Confirm that all work with LCMV and infected animals/samples is covered by approved **biosafety and animal use protocols**.
- Perform all live-virus manipulations (thawing virus stocks, infection steps, handling of unfixed plates) in a **certified Class II biosafety cabinet**.
- Use **dedicated infectious incubators** and clearly label plates, flasks, and waste containers with the virus strain and date.
- Wear appropriate **PPE** at all times (lab coat/gown, gloves, eye/face protection as required).

- Decontaminate all liquid waste (e.g. with freshly prepared bleach) before disposal. Solid waste (tips, plates, tubes) must be autoclaved or otherwise inactivated according to institutional guidelines.
- OPD and its solutions are handled as **chemical hazardous waste**; follow chemical safety rules in addition to biosafety rules.
- Pregnant or immunocompromised personnel should not work with LCMV unless explicitly allowed under institutional policy with additional safeguards.

Version History

Version	Date	Description
v1.0	2025-12-01	Initial LCMV plaque assay SOP.