# 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)
```

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
```

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. |