

LCMV Focus-Forming Assay (Immunofocus)

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Purpose

This protocol describes a **focus-forming assay (FFA)** for determining infectious titers of LCMV stocks using antibody-based detection of infected foci on **Vero cells** with a semi-solid overlay.

Compared with a classic plaque assay, the FFA:

- Uses **immunostaining** (e.g. anti-NP, VL-4) to visualize infected foci.
- Can detect infection **earlier** (typically 48–72 h post infection).
- Is useful for strains or conditions with limited cytopathic effect and for titrating experimental virus stocks.

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.

Approximate timing

- Vero maintenance and expansion: **ongoing (split every 2–3 days as needed)**
- Day –1: Vero seeding for infection (default method): **30–60 min**, depending on number of plates
- Day 0: Virus dilution and infection, overlay addition: **1.5–2.5 h**, depending on number of samples and dilutions
- Day 2–3: Fixation and immunostaining: **3–4 h** hands-on time (spread over incubation steps)
- Focus counting and titer calculation: **30–60 min per plate**, depending on number of foci

Total calendar time: ~**2–3 days** from infection to readout, plus routine cell culture maintenance.

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Principle

- Serial **5-fold** dilutions of virus are prepared in a 24-well dilution plate and used to infect a suspension of Vero cells in a matching 24-well incubation plate.
- After a short incubation (adsorption/infection phase), cells are overlaid with a **semi-solid methylcellulose/2× DMEM mix** and incubated for 60–72 h to allow local spread and formation of discrete foci.
- Cells are fixed, permeabilized, and stained with **primary anti-LCMV antibody** (VL-4, rat anti-NP), followed by an HRP-conjugated goat anti-rat IgG secondary antibody.
- Foci are visualized using an **OPD (o-phenylenediamine)** substrate and counted under a microscope.
- FFU/mL is calculated from focus counts, dilution factor, and inoculum volume.

Materials

Cells and Media

- **Vero cells** (African green monkey kidney)
- **Vero propagation medium** (see `buf_vero_propagation_medium.qmd` for detailed recipe)
- Trypsin-EDTA for passaging Vero cells
- PBS without Ca^{2+} / Mg^{2+}
- Infection/dilution medium (e.g. DMEM + 2% FCS/FBS) for virus dilutions

Overlay and Related Buffers

- **2% methylcellulose** solution (w/v) in dH O, sterilized (e.g. autoclaved)
- **2× DMEM** overlay medium, sterile
- Optional: pre-warmed PBS for washing

Virus

- LCMV stocks (e.g. Armstrong, Clone 13, WE, Docile) stored at -80°C in single-use aliquots

Antibodies and Detection Reagents

- **Primary antibody:**
 - VL-4 (rat anti-LCMV NP), stored and handled according to supplier or in-house conditions
- **Secondary antibody:**
 - Peroxidase AffiniPure Goat Anti-Rat IgG (HRP-conjugated), or equivalent
- **Chromogenic substrate:**
 - OPD (o-phenylenediamine) tablets or powder for HRP-based detection, with appropriate buffer (e.g. citrate-phosphate) and H O

Fixation and Permeabilization

- 4% paraformaldehyde (PFA) in PBS
- Permeabilization buffer:
 - PBS + Triton X-100 (final concentration e.g. 0.1–0.3%)
- Blocking/antibody diluent buffers:
 - 5% FCS in PBS
 - 2% FCS in PBS (for primary and secondary antibody dilutions)

General Reagents and Consumables

- Sterile 24-well tissue culture plates:
 - One or more **incubation plates** for Vero cells
 - Matching **dilution plates** for virus serial dilutions
- Sterile conical tubes (15 mL, 50 mL)
- Microcentrifuge tubes
- Micropipettes and **sterile filtered tips**
- **Serological pipettes** (e.g. 5 mL, 10 mL, 25 mL)
- Waste containers with freshly prepared disinfectant (e.g. 10% bleach)

Equipment

- Class II biosafety cabinet
- **Dedicated infectious CO incubator** set to 37°C, 5% CO
- Inverted light microscope
- Humidified chamber for antibody incubations
- Timer
- Fume hood for OPD preparation and development

Warning

Always use a **designated infectious incubator** for LCMV work. Do not use the same CO incubator for sterile cell culture expansion and virus-infected plates.

Preparation

Vero cell maintenance

- Maintain Vero cells in Vero propagation medium (see `buf_vero_propagation_medium.qmd`) and passage every **2–3 days** when cells reach ~60–80% confluence.
- For routine splitting:
 - Aspirate medium.
 - Wash with ~10 mL PBS.

- Add 5–7 mL trypsin-EDTA and incubate at 37°C for **5–10 min**, monitoring under the microscope until cells detach.
- Neutralize trypsin with 20–30 mL Vero propagation medium.
- Collect cells into a 50 mL tube and centrifuge at **4°C, 5 min, 1500 rpm**.
- Aspirate supernatant and resuspend the pellet in an appropriate volume (e.g. 5–10 mL) of fresh medium.
- Seed into flasks/plates at the desired density for continued expansion.

Cell seeding for FFA

Two seeding strategies are described below. The **default method** seeds Vero cells the day before infection to form an adherent monolayer. The **alternative method** seeds Vero cells as a suspension immediately before infection, as described in the collaborator protocol.

Default method (recommended): Day –1 monolayer seeding

1. One day before infection (**Day –1**), prepare a single-cell suspension of Vero cells from a near-confluent flask.
2. Count cells using a hemocytometer or automated cell counter.
3. Dilute cells in Vero propagation medium to reach **70–90% confluence** in 24-well plates on the day of infection (**Day 0**). As a starting point:
 - Aim for $\sim 1\text{--}2 \times 10^5$ cells per well in 24-well plates.
4. Seed cells in **24-well incubation plates** (e.g. 0.5–1 mL per well).
5. Incubate overnight at 37°C, 5% CO₂ in the infectious incubator.

Alternative method: Same-day seeding and infection (suspension-based)

This method is based on the collaborator’s protocol and may be useful when a tightly controlled cell number per well is desired.

1. On **Day 0**, detach Vero cells from a near-confluent flask as described under maintenance.
2. Resuspend cells in Vero propagation medium and count.
3. Calculate total cell number in the suspension. For example, if a counted sample corresponds to N cells per defined volume, determine the concentration and total cells in the resuspension volume.
4. Dilute the cell suspension to **8×10^5 cells/mL** in Vero propagation medium.
5. Keep the cell suspension on ice until seeding.
6. For each well of the **24-well incubation plate**, add **200 μ L** of this suspension ($\rightarrow 1.6 \times 10^5$ cells per well).
7. Proceed directly with virus addition and infection as described in the infection section.

Tip

The monolayer (Day –1) method may provide slightly more uniform adherence, while the same-day suspension method offers tightly controlled cell numbers per well. Choose one approach and use it consistently within a given experiment.

Overlay media

2% methylcellulose

- Prepare a **2% (w/v)** methylcellulose solution by dissolving 20 g methylcellulose in 1 L dH O.
- Autoclave (if compatible with your methylcellulose product) or sterile-filter if possible.
- Store at 4°C. Warm to room temperature before mixing with 2× DMEM.

2× DMEM overlay medium

Prepare **2× DMEM** as follows (example formulation; adapt to your laboratory's standard buffer documents if applicable):

- 1 bag **2× DMEM powder**
- 10 mL FCS/FBS (to yield 10% final in the overlay)
- 5 mL Pen/Strep (2%)
- 1.1 g NaHCO₃
- dH O to a final volume of **265 mL**

Mix thoroughly and sterile-filter into appropriate storage bottles. Store at 4°C and warm to room temperature before use.

Overlay mixture

- Immediately before use, mix equal volumes of **2× DMEM overlay medium** and **2% methylcellulose** to generate a **1× DMEM, 1% methylcellulose overlay**.
- Keep at room temperature during the overlay step. The solution will be **viscous**.

Tip

Because the methylcellulose overlay is viscous, use a **cut pipette tip** (trimmed with sterile scissors) or a **serological pipette** to avoid bubbles and ensure accurate dispensing.

Virus dilution series (5-fold plate-based)

1. Thaw a virus aliquot rapidly at 37°C and immediately transfer to ice.
2. Prepare a **24-well dilution plate**:
 - Add **400 µL** infection/dilution medium (e.g. DMEM + 2% FCS) to each well required for the serial dilution.
3. For each virus stock:
 - Add **100 µL** virus stock to the first well in the column (top row) to obtain a 1:5 dilution.
 - Mix by pipetting up and down gently.
4. Serial 5-fold dilutions:
 - Transfer **100 µL** from the first well to the next well in the column containing 400 µL medium.

- Mix thoroughly by pipetting.
 - Repeat down the column to achieve the desired range of 5-fold dilutions (e.g. dilutions 5^1 to 5^5 or more, depending on expected titer).
5. Use fresh tips for each transfer to avoid back-contamination.
 6. Keep the dilution plate on ice until infection.

Infection and incubation

1. Ensure Vero cells are prepared according to your chosen seeding method (monolayer or same-day suspension) in the **24-well incubation plate**.
2. For each virus dilution:
 - Transfer **200 μ L** from the corresponding well of the **dilution plate** onto the appropriate well of the **incubation plate**.
 - Each infection well should now contain:
 - **200 μ L** Vero cell suspension (if using same-day method) or the monolayer in existing medium (if using Day -1 method; in that case, aspirate most of the medium first, leaving a thin layer to prevent drying).
 - **200 μ L** of virus dilution.
3. Gently rock the plate to distribute the inoculum evenly.
4. Incubate at 37°C , 5% CO_2 in the infectious incubator for **3 h** (adsorption/infection phase).
5. After 3 h, without aspirating the inoculum, overlay each well with **400 μ L** of the pre-mixed methylcellulose/ $2\times$ DMEM overlay (see above).
 - Use a **cut pipette tip** or **serological pipette** to handle the viscous overlay and avoid bubbles.
6. Return the plates to the infectious incubator and incubate at 37°C , 5% CO_2 for **60–72 h**.
 - Do not disturb the plates during this period to allow discrete foci to form.

Tip

A typical schedule is **Day 0: infection and overlay**, **Day 2–3: fixation and staining**. For example, Monday infection \rightarrow Wednesday or Thursday readout.

Fixation

From this point on, all steps are performed at **room temperature** (unless otherwise specified). Work in the biosafety cabinet until fixation is complete.

1. After 60–72 h incubation, remove plates from the incubator and place them in the biosafety cabinet.
2. Carefully aspirate the overlay and supernatant without touching the cell layer.
3. Add **300–400 μ L** of **4% PFA in PBS** to each well.
4. Incubate for **30 min** at room temperature.
5. Aspirate the fixative into a container containing an appropriate disinfectant (e.g. 10% bleach).
6. Gently wash wells **2–3 \times with PBS**.

Once fixation is complete and the plates have been washed, subsequent steps (permeabilization, antibody staining, OPD development) can be performed outside the BSL-2 cabinet if permitted by local biosafety regulations, but OPD handling must be done in a **fume hood**.

Permeabilization and priming

1. Add **200 μ L** of **Triton X-100 permeabilization solution** (e.g. PBS + 0.1–0.3% Triton X-100) to each well.
2. Incubate for **20 min** at room temperature.
3. Aspirate the permeabilization solution.
4. Add **400 μ L** of **5% FCS in PBS** to each well.
5. Incubate for **~20 min** at room temperature.
6. Aspirate the 5% FCS/PBS solution immediately before adding primary antibody.

Antibody staining

Primary antibody (VL-4)

1. Prepare the primary antibody solution fresh on the day of use:
 - Dilute **VL-4 (rat anti-LCMV NP) 1:400** in **2% FCS in PBS**.
 - Example: **50 μ L VL-4** into **20 mL 2% FCS/PBS** (adjust volumes as needed).
2. Aspirate the 5% FCS/PBS solution from each well.
3. Add **200 μ L** of the VL-4 working solution to each well.
4. Incubate for **60 min** at room temperature in a humidified chamber (or covered to prevent evaporation).
5. After incubation, wash wells **2 \times with PBS** (e.g. 5 min per wash).

Secondary antibody (HRP-conjugated goat anti-rat IgG)

1. Prepare the secondary antibody solution fresh on the day of use:
 - Dilute **Peroxidase AffiniPure Goat Anti-Rat IgG (HRP) 1:100** in **2% FCS in PBS**.
 - Example: **200 μ L** secondary antibody into **20 mL 2% FCS/PBS** (adjust volumes as needed).
2. Add **200 μ L** of the secondary antibody solution to each well.
3. Incubate for **60 min** at room temperature in a humidified chamber.
4. Around **10 min before the end** of the secondary incubation, prepare the OPD substrate solution (see below).
5. After incubation, wash wells **2 \times with PBS** (e.g. 5 min per wash).

OPD development and stopping the reaction

Warning

OPD is hazardous and must be handled in a **fume hood** with appropriate PPE. OPD solutions and contaminated consumables must be collected as **chemical hazardous waste** according to institutional regulations.

1. Prepare the OPD substrate solution fresh approximately **10 min** before use, following the manufacturer's instructions (e.g. OPD tablet in buffer with H_2O).
2. Transfer the fixed, antibody-stained plates to a **fume hood**.
3. Add **400 μ L** of OPD substrate solution to each well.
4. Incubate for **10–20 min** at room temperature, monitoring the development of foci periodically.
 - Stop the reaction once plaques/foci are clearly visible with good contrast but before background staining becomes excessive.
5. When development is complete:
 - Aspirate OPD solution into a designated **chemical waste container** in the fume hood.
6. Wash wells **2 \times with 400 μ L PBS** to remove residual substrate.
7. Plates can be kept in PBS until counting or allowed to air-dry if preferred.

Focus counting and titer calculation

1. Examine wells under a light microscope at low magnification (e.g. 4 \times or 10 \times objective).
2. Identify wells and dilutions that have **10–100 discrete foci** (well-separated, countable).
3. For each suitable well, record:
 - Dilution factor (e.g. $5^3 = 125$)
 - Number of foci
 - Volume of virus inoculum added per well (in mL; e.g. 0.2 mL from the dilution plate)
4. Calculate focus-forming units per mL (FFU/mL)

Formula:

$$\text{FFU/mL} = (\text{Number of foci} \times \text{Dilution factor}) / \text{Volume of inoculum (mL)}$$

Example (5-fold dilution):

- 40 foci in a well
- Inoculated with **0.2 mL** of a **5 dilution** ($5^3 = 125$)

$$\text{FFU/mL} = (40 \times 125) / 0.2 = 125,000 \text{ FFU/mL} = 1.25 \times 10^5 \text{ FFU/mL}$$

5. If multiple wells at the same dilution are counted (technical replicates), calculate the **mean FFU/mL** and optionally the standard deviation.
6. Report titers as FFU/mL and record all raw counts and calculations in your lab notebook or electronic record.

Controls and quality checks

- **Mock-infected wells** (no virus) to assess background staining and non-specific antibody binding.
- **Positive control virus stock** of known titer to monitor assay performance across experiments.
- Wells should exhibit:
 - Minimal background staining in mock-infected controls.
 - Clear, discrete foci in infected wells.
- If background is high or foci are poorly defined:
 - Check permeabilization and blocking conditions.
 - Optimize primary and secondary antibody dilutions.
 - Confirm that overlay viscosity and incubation time are appropriate.

Troubleshooting

Issue	Possible cause	Suggested action
No foci at any dilution	Virus inactive or dilutions too high	Test lower dilutions; verify virus storage and freeze–thaw history
Very high foci numbers in all wells	Virus titer underestimated; dilutions too low	Increase dilution range (more 5-fold steps)
High background staining	Inadequate blocking or washing; antibody too concentrated	Increase blocking time; add more/longer washes; titrate antibodies
Foci too small or faint	Short incubation; suboptimal antibody concentration	Extend incubation (up to 72 h); increase primary or secondary slightly
Foci merging into large patches	Incubation too long; inoculum too concentrated	Shorten incubation; use higher (more dilute) dilutions
Uneven staining or edge effects	Incomplete coverage during antibody or OPD steps	Ensure sufficient volume and gentle rocking; avoid plate drying
Bubbles or holes in overlay	Viscous overlay handled with narrow tips	Use cut tips or serological pipettes; avoid vigorous pipetting

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 focus-forming assay (FFA) SOP (generic, MC57G/Vero, DAB-based).
v1.1	2025-12-02	Updated to Vero-only FFA with methylcellulose overlay, VL-4/OPD staining, and revised timing.