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 ~1–2 × 10 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% CO 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 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 µL** of this suspension (→ **1.6 × 10 cells per well**).
7. Proceed directly with virus addition and infection as described in the infection section.

> **Tip**
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> 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 NaHCO
- 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
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> 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 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 µL** from the corresponding well of the **dilution plate** onto the appropriate well of the **incubation plate**.
   - Each infection well should now contain:
     - **200 µL** 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 µL** of virus dilution.
3. Gently rock the plate to distribute the inoculum evenly.
4. Incubate at 37°C, 5% CO in the infectious incubator for **3 h** (adsorption/infection phase).
5. After 3 h, without aspirating the inoculum, overlay each well with **400 µL** of the pre-mixed methylcellulose/2× 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 for **60–72 h**.
   - Do not disturb the plates during this period to allow discrete foci to form.

> Tip
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> A typical schedule is **Day 0: infection and overlay**, **Day 2–3: fixation and staining**. For example, Monday infection → 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 µL** 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× 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 µL** 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 µL** 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 µL 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 µL** 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× 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 µL** secondary antibody into **20 mL** 2% FCS/PBS (adjust volumes as needed).
2. Add **200 µL** 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× 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 O).
2. Transfer the fixed, antibody-stained plates to a **fume hood**.
3. Add **400 µL** 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× with 400 µL 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× or 10× 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:
FFU/mL = (Number of foci × Dilution factor) / Volume of inoculum (mL)

Example (5-fold dilution):

- 40 foci in a well
- Inoculated with **0.2 mL** of a **5 dilution** (5 = 625)

FFU/mL = (40 × 625) / 0.2 = 125,000 FFU/mL = 1.25 × 10 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.