xCELLigence Real-Time Cytotoxicity Assay for LCMV-Infected Fibroblasts

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Purpose

This protocol describes a real-time impedance-based cytotoxicity assay using the xCELLigence RTCA system to measure the killing activity of cytotoxic effector cells against LCMV-infected and uninfected **C57BL/6J fibroblasts (MEFs)**.

The protocol covers **in vitro LCMV infection** of target fibroblasts and **real-time cytotoxicity measurement** using label-free impedance monitoring. Effector cells are not described here — they should be prepared according to relevant isolation protocols and added as described in **STEP 3**.

Example effectors: NLCs or CD8+ T cells sorted from LCMV-WE infected C57BL/6J mice (NLCs typically at day 3 p.i., CD8+ T cells at day 7 p.i.).

Critical notes (read before starting)

- **xCELLigence E-Plates are required** — standard tissue culture plates cannot substitute.
- **Fibroblasts must be seeded, allowed to adhere, then infected** when at ~50% confluence. After 48 hours of infection, they are harvested, counted, and re-plated into E-Plates.
- **MEFs are routinely cultured in DMEM + 10% FBS + 1% P/S**, but for this assay they are adapted to T cell medium (RPMI + 10% FCS + 1% P/S + 2 mM L-glutamine + 1% NEAAs + 50 uM beta-mercaptoethanol) to match the effector cell environment — see STEP 1 for details.
- **Target cell density in E-Plates is critical** — aim for 60–80% confluence at the time of effector addition, not at seeding.
- **Temperature stability is critical** — pre-warm all media to 37°C before addition to E-Plates.
- **Minimize plate handling** once the assay begins to avoid impedance artifacts.
- **Do not over-confluent MEFs before infection** — cells should be in active growth phase (~50% confluent) for optimal viral uptake.

Approximate timing

Day -3: Fibroblast seeding for infection (**30-60 min**)
Day -1 (48 h later): LCMV infection of fibroblasts (**1-2 hours**)
Day +1 (48 h post-infection): Harvest infected fibroblasts, E-Plate setup, target cell seeding (**2-3 hours**)
Day +2 (24 h later): Effector addition and assay start (**1-2 hours**)
Real-time monitoring: 24-48 hours (continuous)
Data analysis: 1-2 hours

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Materials

Fibroblasts and culture reagents

Target cells:

- C57BL/6J embryonic fibroblasts (primary or immortalized MEFs)

MEF standard culture medium (not used in this assay):

- DMEM high glucose
- 10% FBS
- 1% Penicillin/Streptomycin

MEF killing assay medium (used in this protocol):

- RPMI 1640
- 10% FCS
- 1% Penicillin/Streptomycin
- 2 mM L-glutamine (from 200 mM stock, 1:100)
- 1% Non-essential amino acids (NEAAs)
- 50 uM beta-mercaptoethanol (from 50 mM stock, 1:1000)

Virus infection:

- LCMV-WE stock (titered, stored at -80°C)
- Low-serum adsorption medium: RPMI + 2% FCS (for virus adsorption step)
- Sterile PBS (endotoxin-free)

Passaging:

- 0.25% Trypsin-EDTA (pre-warmed to 37°C)

xCELLigence system

Hardware:

- xCELLigence RTCA instrument (SP, MP, or DP)
- E-Plate 96 (Agilent/ACEA Biosciences)
- Standard CO2 incubator (37°C, 5% CO2)

Plate configuration:

- Each plate: 2 columns x 8 rows = 16 wells per plate
- 3 plates simultaneously = 48 wells total capacity
- Minimum duplicate wells recommended per condition

Software:

- xCELLigence RTCA Software (for data acquisition and %Cytolysis calculation)
- Data analysis: R / GraphPad Prism

Controls and reagents

Reagent	Purpose
Medium only wells	Background impedance
Uninfected MEFs + medium	Spontaneous target death (no effector)
LCMV-infected MEFs + medium	Spontaneous target death in infected targets
Uninfected MEFs + effectors	Non-specific/MHC-unrestricted killing
Triton X-100, 1% in PBS	Maximum lysis control

Procedure

STEP 1 — MEF culture and seeding for infection

Background: MEF culture conditions

MEFs are adherent fibroblasts typically maintained in **DMEM + 10% FBS + 1% P/S** at 37°C, 5% CO2. However, for use in this cytotoxicity assay, MEFs are adapted to T cell medium (**RPMI + 10% FCS + 1% P/S + 2 mM L-glutamine + 1% NEAAs + 50 uM beta-mercaptoethanol + 50 uM beta-mercaptoethanol**) to match the effector cell environment. Adaptation is done by switching culture medium to T cell medium at least one passage (ideally two) before the experiment. All culture and assay steps in this protocol use T cell medium unless otherwise stated. Primary MEFs should not be passaged beyond P4-5, as proliferative capacity decreases. Cells are subcultured at **70-85% confluence** — do not allow full confluence before passaging, as contact inhibition impairs subsequent growth.

MEF seeding density reference

- **Seeding density for culture/expansion:** 2x10^4 – 4x10^4 cells/cm^2
- **T75 flask surface area:** 75 cm^2 — seed 1.5x10^6 – 3x10^6 cells per T75 for routine passaging
- **Expected yield from a confluent T75:** ~10x10^6 – 20x10^6 cells
- **Target density for infection (overnight approach):** seed at a density that gives ~50% confluence 24-48 h later

Option A — Standard procedure (seed day before infection)

1. **Aspirate medium** and wash monolayer with warm PBS (1x).
2. **Add 0.25% Trypsin-EDTA** — for a T75 flask, add ~2-3 mL. For a 6-well plate, 0.3-0.5 mL per well.
3. **Incubate at 37°C for 3-5 min.** Monitor under microscope — do not over-trypsinize (reduces viability).
4. **Neutralize trypsin** by adding 2-3x volume of complete T cell medium (FBS inactivates trypsin).
5. **Transfer to a centrifuge tube** and spin at 300 x g, 5 min, RT.
6. **Aspirate supernatant** and resuspend in complete medium.
7. **Count cells** using trypan blue exclusion.
8. **Seed into standard 6-well plates or T25 flasks** at a density that will yield ~50% confluence within 24-48 h. Infect the following day (STEP 2).

> **Note**
>
> For infection, cells should be actively dividing and at ~**50% confluence** — this is the optimal state for LCMV uptake. Avoid seeding into E-Plates at this stage; E-Plate seeding follows post-infection (STEP 2).

Option B — Same-day trypsin, replate, and infect (compressed timeline)

Use this approach when MEFs are already too confluent (>60-70%) on the intended infection day and there is no time to seed the day before.

1. **Trypsinise** as per Option A steps 1-7.
2. **Count and reseed** into 6-well plates. Seed at a density sufficient to achieve ~50% confluence within 4 hours — seed more cells than the overnight approach since proliferation is not relied upon to reach target density.
3. **Allow 4 hours adherence** at 37°C, 5% CO2.
4. **Confirm attachment** by microscopy before proceeding.
5. **Infect immediately** — proceed to STEP 2. The 1h adsorption step is unchanged.

> **Note**
>
> Same-day replating is acceptable when timeline constraints require it. MEFs adhere within 1-2 h and resume active metabolism within 3-4 h post-trypsin — viral uptake is not meaningfully impaired provided cells are confirmed attached and not blebbing.

STEP 2 — LCMV infection of fibroblasts

Timing: Infect when cells are at ~50% confluence (~24-48 h after seeding). Infection runs for 48 hours.

Virus preparation

1. **Thaw LCMV-WE stock** rapidly in 37°C water bath. Transfer immediately to ice.
2. **Count cells** in the wells to be infected, or estimate based on prior seeding density and expected doubling.
3. **Calculate MOI and required PFU:**

   MOI (multiplicity of infection) is defined as the number of virus particles (PFU) per cell:

   PFU required = MOI x number of cells

   Volume of virus stock = PFU required / stock titer (PFU/mL)

   Example: MOI 0.01, 4 x 10^5 cells, stock titer 1 x 10^8 PFU/mL
   → PFU required = 0.01 x 400,000 = **4,000 PFU**
   → Volume of stock = 4,000 / 1 x 10^8 = **0.04 uL** — dilute into adsorption medium to a workable volume (e.g. 0.5-1 mL per well)
4. **Dilute virus** in low-serum adsorption medium (RPMI + 2% FCS) to the calculated concentration.

> **Note**
>
> A MOI range of **0.01-0.1 PFU/cell** is appropriate for LCMV infection of actively growing fibroblasts at ~50% confluence. **MOI 0.01 has previously been used** for this assay, which gives moderate infection rates and minimal cytopathic effect at 48 h — a reasonable starting point. Higher MOI (up to 0.1) will increase infection efficiency but may cause more target cell death before the assay begins.

Infection procedure

1. **Remove culture medium** from wells.
2. **Add infection volume** (0.5-1 mL per well for 6-well; scale accordingly): virus diluted in adsorption medium.
3. **Add equivalent volume of adsorption medium** (no virus) to control wells.
4. **Incubate 1 hour** at 37°C. Rock plate gently every 15 min to distribute virus evenly.
5. **Remove virus-containing medium** and wash wells **2x with warm PBS**.
6. **Add complete T cell medium** (normal culture volume).
7. **Incubate 48 hours** at 37°C, 5% CO2. This allows viral protein expression and presentation on target cell surface.

> **Warning**
>
> All steps involving live LCMV must be performed in a certified Class II biosafety cabinet. Decontaminate all liquid waste with freshly prepared bleach before disposal. Follow institutional BSL-2 guidelines.

STEP 3 — Harvest infected fibroblasts and seed E-Plates

Timing: 48 hours post-infection.

Fibroblast harvest

1. **Visually inspect wells** under microscope — infected MEFs should appear morphologically intact but may show subtle changes. Significant cytopathic effect at this stage indicates infection was too high (reduce MOI).
2. **Aspirate medium** from all wells (infected and uninfected controls).
3. **Wash once with warm PBS.**
4. **Add pre-warmed 0.25% Trypsin-EDTA** (0.3-0.5 mL per well of a 6-well plate).
5. **Incubate 3-5 min at 37°C.** Monitor — do not over-trypsinize infected cells, which may be more fragile.
6. **Neutralize with complete T cell medium** (2-3x trypsin volume).
7. **Transfer to 15 mL tube** and centrifuge at 300 x g, 5 min, RT.
8. **Resuspend in complete T cell medium** and count. Assess viability by trypan blue exclusion — aim for >85% viability.

E-Plate background measurement

1. **Add 50 uL complete T cell medium** to each well of the E-Plate.
2. **Place plate in xCELLigence** instrument.
3. **Perform background read** to establish baseline impedance.
4. **Remove plate** from instrument and proceed immediately to cell seeding.

Target cell seeding into E-Plates

Choosing target cell number: Prior lab xCELLigence experiments (CD160, B16F10/MCA1956 targets) used **25,000 cells/well**. However, given that effector cell numbers are limiting in this assay, reducing target cell number is desirable to keep E:T ratios achievable. Published literature supports lower seeding densities: an optimization study using porcine fibroblasts as targets found **7,500 cells/well** generated a robust Cell Index within 10 hours (Galli et al., 2013, *Xenotransplantation*). A validated range for adherent targets in 96-well E-Plates across published xCELLigence cytotoxicity protocols is broadly **7,500-25,000 cells/well**.

Recommended starting point for MEFs in this assay: 10,000-15,000 cells/well. This reduces effector demand approximately 2-fold compared to 25,000 targets while maintaining sufficient impedance signal. Validate CI at 24 h before committing to a final density.

1. **Adjust cell concentration** to the target density in complete T cell medium (e.g. 2 x 10^5 cells/mL for 10,000 cells/well in 50 uL).
2. **Add 50 uL per well** to E-Plate wells (total volume = 100 uL with background medium already present).
3. **Assign wells:**
    - LCMV-infected MEFs (effector wells + target-alone control wells)
    - Uninfected MEFs (effector wells + target-alone control wells)
    - Medium-only wells (background)
4. **Allow plate to sit at room temperature in hood for 30 min** — permits cell settling before scanning begins.
5. **Transfer to xCELLigence instrument** and begin scanning (every 15 min).
6. **Monitor target cell adherence** for 24 h before adding effectors.

> **Note**
>
> Target cells should reach a Cell Index of ~1.5-2.5 at the time of effector addition, corresponding to approximately 60-80% confluence. MEFs spread well and may reach this CI faster than tumor lines. If cells over-confluent before 24 h, reduce seeding density for future experiments.

STEP 4 — Effector cell addition and cytotoxicity assay

Timing: 24 hours after E-Plate seeding (once targets are 60-80% confluent).

Effectors should be prepared according to the appropriate isolation protocol and brought to the required cell concentration immediately before use. Examples of typical effectors for this assay:

- **NLCs** sorted or enriched from liver of LCMV-WE infected mice, day 3 p.i.
- **CD8+ T cells** sorted or enriched from spleen of LCMV-WE infected mice, day 7 p.i.

E:T ratio guidance

Published xCELLigence cytotoxicity assays using enriched NK or CD8+ T cells against adherent targets typically span **1:1 to 10:1** for sorted/enriched primary effectors, with higher ratios (up to 25:1–50:1) used for bulk mixed populations (e.g. splenocytes, PBMCs). For sorted NLCs or CD8+ T cells, the biologically informative range is generally **1:1 to 10:1**; ratios above this are useful for establishing the assay ceiling but are not always necessary with potent effectors.

Recommended E:T ratios to test in this assay:

E:T ratio	Context
10:1	Upper range for sorted primary effectors
5:1	Standard; good mid-point
2.5:1	Low-moderate
1:1	Lower limit; sensitivity check

If effector numbers allow, extending to 20:1 or 25:1 provides useful upper-bound data.

Effector cell concentration calculation

Effectors are added in **100 uL at 2x concentration** (final well volume = 150 uL after addition to 50 uL remaining medium).

The tables below give 2x stock concentrations for two recommended target densities. Use the column matching your chosen target number.

For 10,000 target cells/well:

E:T ratio	Effectors/well	2x stock concentration
10:1	100,000	2.0 x 10^6 /mL
5:1	50,000	1.0 x 10^6 /mL
2.5:1	25,000	5.0 x 10^5 /mL
1:1	10,000	2.0 x 10^5 /mL

For 15,000 target cells/well:

E:T ratio	Effectors/well	2x stock concentration
10:1	150,000	3.0 x 10^6 /mL
5:1	75,000	1.5 x 10^6 /mL
2.5:1	37,500	7.5 x 10^5 /mL
1:1	15,000	3.0 x 10^5 /mL

For 25,000 target cells/well (original density, for reference):

E:T ratio	Effectors/well	2x stock concentration
10:1	250,000	5.0 x 10^6 /mL

E:T ratio	Effectors/well	2x stock concentration
5:1	125,000	2.5 x 10^6 /mL
2.5:1	62,500	1.25 x 10^6 /mL
1:1	25,000	5.0 x 10^5 /mL

Effector addition

1. **Pause impedance monitoring** in xCELLigence software.
2. **Remove E-Plate** from instrument.
3. **Pre-warm effector suspensions** to 37°C (place tubes briefly in water bath or incubator).
4. **Add 100 uL effector suspension** per well at 2x concentration. Pipette gently — do **not** mix or disturb the target monolayer.
5. **Add 100 uL complete medium** to target-alone control wells (no effectors).
6. **Add 100 uL Triton X-100 (2% stock)** to maximum lysis control wells (final 1%).
7. **Return plate to instrument** and resume monitoring for 24-48 hours.

> ⚠ Warning
>
> Do not mix wells after effector addition. Pipetting vigorously against the monolayer will disrupt impedance and create artifacts indistinguishable from cytotoxicity.

STEP 5 — Real-time monitoring

Monitoring parameters:

- Data collection interval: every 15 min
- Total duration: 24 h (extendable to 48 h)
- Cytotoxicity typically begins 1-4 h post-effector addition

What to observe:

- Stable CI in target-alone wells = healthy monolayer, assay running correctly
- Decreasing CI in effector-containing wells = cytotoxicity
- Rapid CI drop in Triton wells = expected; confirms instrument sensitivity

Buffers Required

Full composition, preparation, storage, and safety information for each buffer lives in its own linked buffer document — only a concise per-buffer summary is reproduced here, generated automatically from that document.

None

Data analysis

Cytotoxicity calculation

Standard formula adapted for impedance-based assays:

% Specific Lysis = ((CI_target - CI_experimental) / (CI_target - CI_maximal)) x 100

Where:

- **CI_target** = Cell Index of target cells alone at each timepoint
- **CI_experimental** = Cell Index of target + effector wells
- **CI_maximal** = Cell Index of Triton X-100 treated wells (complete lysis)

Data processing steps

1. Export raw data from xCELLigence software.
2. Normalize Cell Index to the time of effector addition (t = 0).
3. Calculate % specific lysis at multiple time points (e.g. 4 h, 12 h, 24 h).
4. Generate dose-response curves: % lysis vs. E:T ratio.
5. Statistical analysis in R or GraphPad Prism.

Key parameters to report

- Time to 50% lysis (LT50) for each E:T ratio
- Maximum cytotoxicity at 24 h
- Area under the curve (AUC) for kinetic analysis
- EC50 (E:T ratio causing 50% lysis)

Quality control criteria

Parameter	Acceptable range
Target cell CI at effector addition	1.5-2.5
CV between replicate wells	<15%
Spontaneous lysis (targets alone) at 24 h	<10%
Maximum lysis (Triton) at 4 h	>90%

Experimental design and plate layout

Required controls

Every run must include the following. These are non-negotiable for data interpretation:

- **LCMV-infected MEFs alone** — spontaneous death of infected targets; required to calculate specific lysis
- **Uninfected MEFs alone** — spontaneous death baseline for uninfected targets

- **Uninfected MEFs + effectors** — non-specific / MHC-unrestricted killing; should be low
- **Medium only** — background impedance; floor reference
- **Triton X-100 (1% final)** — maximum lysis; required for % specific lysis formula
- **Effector cells alone** — confirms effectors do not adhere and contribute artifactually to CI; run during initial assay validation

All controls in duplicate minimum.

Plate maps

Each E-Plate is **2 columns (1–2) × 8 rows (A–H) = 16 wells**. The instrument holds 3 plates simultaneously (48 wells total). Fill in conditions below before each experiment.

PLATE 1

	Col 1	Col 2
A		
B		
C		
D		
E		
F		
G		
H		

PLATE 2

	Col 1	Col 2
A		
B		
C		
D		
E		
F		
G		
H		

PLATE 3

	Col 1	Col 2
A		
B		
C		
D		
E		
F		
G		
H		

Replication

- Minimum duplicate wells per condition (one row = one condition in duplicate)
- Minimum 3 independent experiments using different mouse donors
- Always include target-alone controls for both infected and uninfected conditions on every plate

Troubleshooting

Problem	Possible cause	Solution
High spontaneous lysis	Over-confluence or poor MEF health post-infection	Reduce seeding density; check post-infection viability

Problem	Possible cause	Solution
No cytotoxicity	Effector dysfunction, wrong timepoint, too low E:T	Verify effector activation; increase E:T ratio; check infection efficiency
Variable impedance between replicates	Temperature fluctuations; uneven seeding	Pre-warm all reagents; mix cell suspension well before seeding each well
Poor target adhesion	Suboptimal cell density; trypsin damage	Optimize seeding density; do not over-trypsinize
CI drop in target-alone wells	Overconfluence and detachment; infected cell death	Reduce seeding density; confirm post-infection viability
High background noise	Electrical interference; bubbles in wells	Check instrument calibration; centrifuge E-Plate briefly before scanning

Safety

- Handle LCMV according to institutional BSL-2 biosafety guidelines in a certified Class II biosafety cabinet.
- Decontaminate all liquid waste with freshly prepared 10% bleach before disposal. Autoclave solid infectious waste.
- Wear appropriate PPE (lab coat, gloves, eye protection) throughout.
- Label all infectious plates, tubes, and waste containers with virus strain and date.
- xCELLigence instrument: ensure proper grounding; avoid liquid contact with electronics.

Protocols used

- Effector isolation protocol (NLC or CD8+ T cell, as applicable) — refer to lab SOP
- LCMV-WE stock production — `MUS-LCMV-PROD`

Version history

Version	Date	Author	Change summary
v1.0	2026-03-18	Dillon Corvino	Initial xCELLigence LCMV cytotoxicity protocol

Version	Date	Author	Change summary
v1.1	2026-03-26	Dillon Corvino	Removed effector isolation sections; corrected infection/seeding workflow to seed-infect-harvest-re-plate sequence; added MEF culture conditions and trypsin passaging; added LCMV MOI guidance for fibroblasts; clarified E:T concentration table
v1.2	2026-03-26	Dillon Corvino	Added MOI calculation formula with worked example; noted MOI 0.01 as reference starting point; updated MOI range note
v1.3	2026-03-26	Dillon Corvino	Reduced recommended target cell density to 10,000-15,000 cells/well to reduce effector demand; added literature context (Galli 2013) and comparison to prior lab density (25,000); updated E:T ratio guidance to 1:1-10:1 range for sorted effectors; added three-density concentration calculation tables
v1.4	2026-03-26	Dillon Corvino	Replaced list-based plate layout with blank visual plate maps (3x E-Plate grids, 2 col x 8 row); added required controls section

Version	Date	Author	Change summary
v1.5	2026-04-13	Dillon Corvino	Added MEF seeding density reference ($2x10^{4-4x10}4$ cells/cm^2, T75 yield ~10-20x10^6); restructured STEP 1 into Option A (standard overnight) and Option B (same-day trypsin-replate-infect compressed timeline)
v1.6	2026-04-15	Dillon Corvino	Changed MEF culture medium from DMEM to T cell medium (RPMI + 10% FCS + 1% P/S + 2 mM L-glutamine + 1% NEAAs + 50 uM beta-mercaptoethanol) throughout; added adaptation rationale to STEP 1 background; updated adsorption medium to RPMI-based; Materials section now distinguishes standard DMEM culture from killing assay medium
v1.7	2026-09-18	Dillon Corvino	Replaced `# Buffers used` with an auto-generated `# Buffers Required` section (one heading + one small table per buffer, sourced from each buffer's own `summary:` data), moved to the correct position after Procedure, and fixed leaked R package-startup warnings.