CellTiter-Fluor™ Cell Viability Assay

96-Well Plate Protocol for Cell Plating and Viability Measurement

Assay Overview

The CellTiter-Fluor™ Cell Viability Assay is a nonlytic, fluorescence-based assay that measures the relative number of live cells by detecting a conserved protease activity within intact viable cells. The fluorogenic substrate (GF-AFC) enters living cells where it is cleaved by the live-cell protease to generate a fluorescent signal proportional to the number of viable cells.

SAFETY & IMPORTANT NOTES

- Always wear appropriate PPE: lab coat, gloves, eye protection
- Work in a biological safety cabinet when handling cells
- DMSO is present in the substrate - handle with care
- Shield plates from ambient light during incubation
- Do not incubate longer than 3 hours

⏱️ Time Required

- Plate setup: 30-60 min
- Treatment period: Variable
- Assay incubation: 1.5-2 hours
- Reading: 5-10 min

🔬 Key Features

- Nonlytic assay
- Single reagent addition
- High sensitivity
- Compatible with multiplexing

📊 Applications

- Drug cytotoxicity screening
- Cell proliferation assays
- Compound testing
- Dose-response curves

> ⚠️ **Critical: Edge Effect Prevention**
>
> **Always fill the perimeter wells of the 96-well plate with PBS only (no cells).** This prevents edge effects caused by differential evaporation and temperature gradients that can affect the outer wells. Only the inner 60 wells (rows B-G, columns 2-11) should contain cells and experimental conditions.

Assay Principle

1. **Substrate Entry:** Cell-permeant GF-AFC substrate crosses the membrane of viable cells
2. **Enzymatic Cleavage:** Live-cell protease cleaves the substrate to release fluorescent AFC
3. **Signal Generation:** Fluorescence intensity is proportional to the number of viable cells
4. **Dead Cell Exclusion:** Dead cells with compromised membranes lose protease activity and don't generate signal

Detection Parameters:

- **Excitation:** 380-400 nm
- **Emission:** 505 nm
- **Readout:** Fluorescence (RFU - Relative Fluorescence Units)

Required Materials

CellTiter-Fluor™ Assay Components

- CellTiter-Fluor™ Assay Buffer (Promega)
- GF-AFC Substrate, 100 mM in DMSO (Promega)

- Product Cat.# G6080, G6081, or G6082

Storage: Store at -20°C. Thaw completely before use.

Equipment

- Biological safety cabinet (BSC)
- CO_2 incubator (37°C, 5% CO_2)
- Fluorescence plate reader with appropriate filters
- Multichannel pipettor (8- or 12-channel)
- Reagent reservoir
- Orbital plate shaker (optional)
- Hemocytometer or cell counter

Cell Culture Supplies

- 96-well tissue culture plates (clear or black-walled, clear bottom)
- Cells in culture (trypsinized and counted)
- Complete cell culture medium
- Sterile PBS (for perimeter wells)
- Trypsin-EDTA (for cell harvest)
- Serological pipettes (5, 10, 25 mL)
- Sterile reagent reservoirs

Additional Reagents

- Test compounds/treatments
- Vehicle controls (DMSO, ethanol, etc.)
- Positive cytotoxicity control (optional)
- 0.4% Trypan blue (for viability check)
- 70% ethanol (for disinfection)

Lab-Specific Reagent Preparation

Working Reagent Dilution (Lab Protocol):

In our lab, we use a **1:1000 dilution** of the CellTiter-Fluor™ reagent:

- Dilute the GF-AFC substrate **1:1000** in CellTiter-Fluor™ Assay Buffer
- Example: 10 µL substrate + 10 mL buffer
- Add **25 µL** of this diluted reagent per well
- Each well contains 200 µL media + cells before reagent addition

96-Well Plate Preparation

Step 1: Fill Perimeter Wells with PBS

Mark Done

CRITICAL STEP - Edge Effect Prevention

Always fill perimeter wells with PBS to minimize evaporation and temperature gradient effects. This significantly improves data quality and reproducibility.

1. Take a sterile 96-well tissue culture plate
2. Using a multichannel pipettor, add **200 µL sterile PBS** to:
   - **Row A** (wells A1-A12) - entire top row
   - **Row H** (wells H1-H12) - entire bottom row
   - **Column 1** (wells B1-G1) - left column (excluding corners)
   - **Column 12** (wells B12-G12) - right column (excluding corners)
3. Total perimeter wells: 36 wells filled with PBS
4. Working wells available: 60 wells (rows B-G, columns 2-11)

96-Well Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	**PBS (200 µL)**											
B	**PBS**	Cells + Media (200 µL)										**PBS**
C	**PBS**	Cells + Media (200 µL)										**PBS**
D	**PBS**	Cells + Media (200 µL)										**PBS**
E	**PBS**	Cells + Media (200 µL)										**PBS**
F	**PBS**	Cells + Media (200 µL)										**PBS**
G	**PBS**	Cells + Media (200 µL)										**PBS**
H	**PBS (200 µL)**											

- [ ] PBS only (perimeter wells)
- [ ] Cells + media (working wells)

Step 2: Prepare Cell Suspension

Mark Done

1. Harvest cells by trypsinization (see Cell Culture Protocol)
2. Neutralize trypsin with complete medium containing serum
3. Collect cell suspension and centrifuge at 200-300 × g for 5 minutes
4. Aspirate supernatant and resuspend pellet in fresh complete medium
5. Count cells using hemocytometer or automated cell counter
6. Check viability using trypan blue exclusion (should be ≥90%)
7. Calculate cell concentration and dilute to desired density

Recommended Cell Densities (per well in 200 µL):

Cell Type	Typical Density	Notes
Fast-growing adherent	2,000-5,000 cells/well	Allow 24-48 hr to attach
Slow-growing adherent	5,000-10,000 cells/well	Allow 48-72 hr to attach
Suspension cells	10,000-20,000 cells/well	Plate immediately

⚠️ Optimize cell density for your specific cell line and experimental timeframe

Step 3: Plate Cells in Working Wells

Mark Done

1. Mix cell suspension thoroughly by gentle pipetting (avoid bubbles)
2. Transfer cell suspension to sterile reagent reservoir
3. Using multichannel pipettor, add **200 µL** of cell suspension to working wells
   - Inner 60 wells only (rows B-G, columns 2-11)
   - Do NOT add cells to perimeter wells (already contain PBS)
4. Mix cell suspension frequently to ensure even distribution
5. Gently tap sides of plate to ensure cells are evenly distributed
6. Check wells under microscope to verify even cell distribution
7. Label plate with:
   - Cell line name and passage number
   - Cell density
   - Date and time
   - Your initials

8. Place plate in 37°C, 5% CO_2 incubator

9. Allow cells to attach and equilibrate:
    - Adherent cells: 4-24 hours
    - Suspension cells: 1-2 hours

> **Important Tips:**
> • Pipette cells gently to avoid shearing stress
> • Work quickly but carefully to prevent cell settling in reservoir
> • Avoid introducing bubbles (interfere with readings)
> • For adherent cells, do not disturb plate during attachment period

Step 4: Add Treatments (Optional)

Mark Done

If testing compounds or treatments:

1. After cells have attached/equilibrated, add test compounds

2. Prepare compound dilutions in appropriate solvent (DMSO, ethanol, etc.)

3. Add compounds to achieve desired final concentration in 200 µL total volume

4. Include appropriate controls:
    - **Vehicle control:** Cells + solvent only (no compound)
    - **Untreated control:** Cells in media only
    - **Positive control:** Known cytotoxic compound (optional)
    - **Media only:** No cells (background control)

5. Perform treatments in triplicate or more replicates

6. Gently mix plate after adding compounds (orbital shaker, 30 seconds)

7. Return plate to incubator for desired treatment duration

Example Plate Layout with Treatments:

• Columns 2-3: Untreated controls
• Columns 4-5: Vehicle controls
• Columns 6-11: Compound treatments (serial dilutions or different compounds)
• Include media-only wells for background subtraction

CellTiter-Fluor™ Assay Procedure

Step 1: Prepare CellTiter-Fluor™ Working Reagent

Mark Done

🔬 **Lab-Specific Protocol: 1:1000 Dilution**

In our lab, we use a 1:1000 dilution and add only 25 µL per well (instead of the standard equal volume method). This conserves reagent while maintaining excellent sensitivity.

1. Remove CellTiter-Fluor™ components from -20°C freezer
2. Thaw completely in 37°C water bath
3. Vortex GF-AFC substrate thoroughly to ensure homogeneity
4. Briefly centrifuge substrate tube to recover all volume
5. Prepare working reagent with **1:1000 dilution**:

For one 96-well plate (60 working wells + ~10% extra):

• Calculate total volume needed: 60 wells × 25 µL = 1,500 µL
• Add 10% extra: 1,500 µL × 1.1 = 1,650 µL total
• **Add 1.65 µL GF-AFC Substrate to 1.65 mL Assay Buffer**
• Final dilution: 1:1000

Alternative volumes:

• Small batch: 10 µL substrate + 10 mL buffer (for multiple plates)
• Exact for 1 plate: 1.65 µL substrate + 1.65 mL buffer

6. Mix by vortexing until substrate is thoroughly dissolved
   - Solution may initially appear "milky" - this is normal
   - Continue vortexing until completely dissolved
7. Transfer diluted reagent to sterile reagent reservoir
8. Protect from light (cover with foil or use amber container)

Storage of Prepared Reagent:

• Use within 24 hours if stored at room temperature
• Can store at 4°C for up to 7 days
• Always protect from light
• Do not freeze once diluted

Step 2: Add CellTiter-Fluor™ Reagent to Wells

Mark Done

1. Remove plate from incubator
2. Check cells briefly under microscope (optional):
   - Verify cell morphology
   - Check for contamination
   - Ensure cells are attached (for adherent cells)

3. Using multichannel pipettor, add **25 µL** of CellTiter-Fluor™ working reagent to each well
   - Add to ALL wells
   - Pipette gently down the side of wells to avoid disturbing cells
   - Work systematically (row by row or column by column)
   - Final volume per well: 225 µL (200 µL + 25 µL reagent)
4. Mix plate briefly:
   - Option A: Orbital shaker at low speed for 30 seconds
   - Option B: Gently tap sides of plate
   - Avoid creating bubbles or splashing between wells

> **IMPORTANT:**
> • Work quickly to minimize time cells are outside incubator
> • Do not create bubbles in wells (interferes with fluorescence reading)
> • If bubbles form, pop them with sterile pipette tip or leave to settle

Step 3: Incubate Plate

Mark Done

> 🔬 **Lab Protocol: 1.5-2 Hour Incubation**
>
> In our lab, we incubate for **1.5-2 hours at 37°C** in the cell culture incubator before reading. This provides optimal signal development with our 1:1000 dilution.

1. Cover plate with lid
2. Wrap plate in aluminum foil to protect from light
   - Light exposure can degrade the fluorescent product
   - Ensure foil doesn't interfere with gas exchange
3. Place plate in **37°C, 5% CO_2 incubator**
4. Incubate for **1.5 to 2 hours**
   - Optimal time may vary by cell line - optimize if needed
   - Minimum incubation: 1.5 hours
   - Maximum incubation: 3 hours (do not exceed)
5. Ensure plate remains level and undisturbed during incubation
6. Set timer to avoid over-incubation

Alternative Incubation Conditions:

Temperature	Time	Notes
37°C (recommended)	1.5-2 hours	Optimal protease activity
Room temperature	2-3 hours	Longer time needed, less sensitive

Temperature	Time	Notes
37°C (short)	30-60 min	Lower signal, less optimal

⚠️ Incubation at 37°C in CO_2 incubator is strongly recommended

⏰ **Timing Tips:**

- Set up plate reader during incubation period
- Ensure fluorometer is warmed up and ready
- Prepare plate reader protocol (filters, gain settings)
- Have data analysis template ready

Plate Reading and Data Analysis

Fluorometer Settings

Mark Done

Fluorescence Detection Parameters:

Fluorophore	AFC (Aminofluorocoumarin)
Excitation Wavelength	**380-400 nm**
Emission Wavelength	**505 nm**
Readout	Fluorescence Intensity (RFU)
Read Mode	Top read (recommended) or bottom read

1. Turn on fluorescence plate reader and allow to warm up (10-15 minutes)
2. Set excitation filter to 380-400 nm
3. Set emission filter to 505 nm
4. Adjust gain/sensitivity settings:
   - Start with automatic gain optimization
   - Or use manual gain based on positive control wells
   - Aim for highest signal without saturation
5. Set integration time (typically 0.5-1 second)
6. Configure plate layout in reader software

Important:

> Deviation from optimal filter settings may adversely affect assay sensitivity and performance. If exact filters are not available, use the closest available wavelengths.

Reading the Plate

Mark Done

1. After 1.5-2 hour incubation, remove plate from incubator
2. Remove foil covering (plate can now be exposed to ambient light briefly)
3. Check for bubbles in wells:
   - If bubbles present, try to pop with sterile pipette tip
   - Or let plate sit 2-3 minutes for bubbles to dissipate
   - Bubbles can interfere with fluorescence reading
4. Wipe bottom of plate with lint-free cloth or Kim-wipe (if bottom-read)
5. Place plate in fluorescence reader
6. Ensure plate is properly aligned and seated
7. Start reading protocol
8. Save raw data immediately after reading

Reading Tips:

- Read all plates from same experiment with same instrument settings
- Record all settings (gain, integration time, filters) for reproducibility
- If signal is too weak, you can re-incubate plate for 30-60 more minutes
- If signal is saturated, dilute samples and re-read (or adjust gain if possible)

Data Analysis

Mark Done

Step 1: Background Subtraction

1. Calculate average fluorescence of media-only wells (no cells)
2. Subtract this background from all sample wells
3. Formula: `Corrected RFU = Sample RFU - Average Background RFU`

Step 2: Calculate Viability (Relative to Control)

Express data as percentage of untreated control:

```
% Viability = (Treated Sample RFU / Average Control RFU) × 100
```

Where Control RFU = average fluorescence of untreated/vehicle control wells

Step 3: Statistical Analysis

- Calculate mean and standard deviation for replicates
- Perform appropriate statistical tests (t-test, ANOVA, etc.)
- Calculate IC_{50} values if dose-response curves
- Assess data quality (CV%, Z-factor if applicable)

Step 4: Quality Control Checks

- ✓ Untreated control wells show high, consistent signal
- ✓ Background (no-cell) wells show low signal
- ✓ Signal-to-background ratio ≥ 3:1 (preferably ≥10:1)
- ✓ CV% between replicates < 20%
- ✓ Perimeter PBS wells excluded from analysis
- ✓ No obvious edge effects in working wells

Expected Results:

• **Viable cells:** High fluorescence signal (proportional to cell number)

• **Dead/dying cells:** Low fluorescence signal (loss of protease activity)

• **Dose-response:** Decreasing fluorescence with increasing cytotoxic compound concentration

• **Linear range:** Signal proportional to cell number from ~500-10,000 cells/well

Troubleshooting Guide

Common Issues and Solutions

Problem	Possible Cause	Solution
Low or no signal	• Too few cells • Cells not viable • Insufficient incubation • Wrong filters/settings	• Increase cell density • Check cell viability before plating • Extend incubation to 2-3 hours • Verify excitation (380-400nm) and emission (505nm) filters

Problem	Possible Cause	Solution
High background signal	• Serum contains protease activity • Media fluorescence • Contamination	• Use low-protease serum lot • Include media-only wells for background subtraction • Check for bacterial/fungal contamination
Edge effects	• Perimeter wells not filled with PBS • Evaporation • Temperature gradients	• Always fill perimeter with PBS (critical!) • Use humidified incubator • Avoid plate stacking in incubator • Allow plate to equilibrate before reading
High variability between replicates	• Uneven cell distribution • Bubbles in wells • Poor mixing of reagent	• Mix cell suspension frequently during plating • Remove bubbles before reading • Mix reagent thoroughly; vortex before use • Mix plate gently after reagent addition
Signal saturation	• Too many cells • Gain too high • Over-incubation	• Reduce cell density • Decrease instrument gain • Reduce incubation time • Optimize cell number for linear range
Unexpected cell death in controls	• DMSO toxicity • Reagent toxicity • Prolonged incubation	• Keep DMSO < 0.5% final concentration • The assay reagent is nonlytic - should not kill cells • Do not exceed 3 hour incubation
No difference between treated and untreated	• Compound not cytotoxic • Wrong concentration • Insufficient treatment time	• Verify compound activity with known positive control • Test wider concentration range • Extend treatment duration • Check compound solubility
Precipitate in wells	• Substrate not fully dissolved	• Vortex reagent thoroughly before use • Ensure complete thawing at

Problem	Possible Cause	Solution
	• Compound precipitation	37°C • Use appropriate compound solvents • Pre-dissolve compounds properly

Optimization Recommendations

For New Cell Lines:

- Perform cell number titration (500-10,000 cells/well)
- Test incubation time (30 min, 1 hr, 1.5 hr, 2 hr, 3 hr)
- Determine optimal seeding density and assay timepoint
- Establish signal-to-background ratio for your cell line

For New Compounds:

- Test multiple concentrations (dose-response)
- Include vehicle control at highest solvent concentration
- Test multiple timepoints if kinetics unknown
- Check for compound fluorescence interference

Best Practices Summary:

✓ Always fill perimeter wells with PBS
✓ Use cells with >90% viability
✓ Protect plate from light during incubation
✓ Include appropriate controls (untreated, vehicle, media-only)
✓ Perform experiments with technical replicates (n≥3)
✓ Maintain consistent timing between replicates
✓ Record all instrument settings for reproducibility
✓ Store prepared reagent protected from light

Protocol adapted from Promega CellTiter-Fluor™ Technical Bulletin TB371

Lab-specific modifications: 1:1000 dilution, 25 µL/well, 1.5-2 hr incubation