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Life science solutions

Cell Viability and Cytotoxicity Kit, with Calcein AM and EthD-1

Contents

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Cell Viability and Cytotoxicity Kit with Calcein AM and EthD-1 is a ready-to-use kit designed for the simultaneous assessment of cell viability and cytotoxicity via dual-fluorescence staining that distinguishes viable and non-viable mammalian cells using two indicators of cell status: intracellular esterase activity and plasma membrane integrity.

The kit contains two complementary probes:

- **Calcein AM** is a nonpolar, cell-permeant fluorogenic probe. Once inside live cells, it is hydrolyzed by intracellular esterases to produce bright green-fluorescent Calcein, which is retained in cells with intact membranes. Fluorescence intensity correlates with metabolic activity and the number of viable cells.
- **Ethidium homodimer-1 (EthD-1)** is a high-affinity nucleic acid intercalating dye that is impermeant to live cells due to its relatively large molecular size and dicationic nature. In cells with compromised membrane integrity, EthD-1 passively enters the cytoplasm and nucleus, where it intercalates into double-stranded nucleic acids (DNA and RNA), resulting in a strong enhancement of fluorescence. The dye serves as an indicator of dead cells and cells in the late stages of apoptosis.

Upon co-incubation with cell cultures, viable cells exhibit green fluorescence (Calcein), whereas dead and apoptotic cells fluoresce red (EthD-1). Minimal spectral overlap of these probes enables two-channel analysis without complex spectral compensation. Background fluorescence is generally low because both probes exhibit relatively weak fluorescence prior to the relevant intracellular conversion or nucleic acid binding.

The assay is suitable for fluorescence microscopy, fluorescence microplate readers, and flow cytometry. It can be adapted to a wide range of eukaryotic cell types, although dye concentrations and incubation times should be optimized for each cell type and instrument.

The assay does not directly measure every form of cell death. Cellular states that do not substantially alter intracellular esterase activity or membrane integrity may not be accurately classified.

Before You Begin

- Use a validated saline buffer. Phenol red and other colored additives can contribute to background or spectral interference and should be evaluated experimentally.
- A suitable D-PBS formulation for the assay is: KCl 200 mg/L, KH_2PO_4 200 mg/L, NaCl 8 mg/L, and Na_2HPO_4 1,15 mg/L.
- Other validated saline buffers may also be used provided that they do not interfere with fluorescence or cell viability.
- Allow frozen stocks to reach room temperature and briefly centrifuge before opening.
- Calcein AM is moisture-sensitive. Minimize exposure of Calcein AM to atmospheric moisture. Reseal the stock immediately after use.
- Prepare aqueous Calcein AM working solutions immediately before the experiment. Do not store them for subsequent experiments.
- EthD-1 is considerably less sensitive to moisture and may be kept as a frozen stock solution when tightly sealed.
- Optimal concentrations depend on cell type, cell density, incubation temperature, optical filters, and instrument sensitivity. In general, use the lowest concentrations that provide a robust separation between live and dead populations. Typical dye concentrations for different applications:

Application	Calcein AM	EthD-1	Starting incubation
Microscopy	0.1–10 µM; often ~2 µM	0.1–10 µM; often ~4 µM	30–45 min, RT
Microplate	0.1–5 µM; often ~1 µM	0.1–10 µM; often ~2 µM	30–45 min, RT
Flow cytometry	100 nM	8 µM	15–20 min, RT

- For plate-reader assays, maintain consistent cell number, reagent volume, incubation time, temperature, and plate position.
- Include live, dead, single-color, and cell-free controls whenever quantitative fluorescence measurements are required.
- In flow cytometry, perform compensation with single-color controls and establish gates using appropriate untreated and killed controls.
- Do not interpret a Calcein-negative/EthD-1-negative population automatically as viable; weak staining can result from insufficient dye loading, low esterase activity, cell type, or technical factors.
- The assay primarily reports esterase activity and membrane integrity. Complementary assays may be required to characterize specific mechanisms of cell death.

Fluorescence Microscopy Protocol

Cell Preparation

1. Grow adherent cells on sterile glass coverslips or another suitable imaging surface. Subconfluent cultures are generally preferable for single-cell assessment.
2. For non-adherent cells, wash the suspension gently with approximately 500–1000 volumes of tissue-culture-grade D-PBS and collect the cells by centrifugation.
3. For adherent cells, wash gently with approximately 500–1000 volumes of D-PBS before staining. This reduces extracellular esterase activity contributed by serum-containing media.
4. Apply the desired cytotoxic treatment before or during staining according to the experimental design.

Dye Concentration Optimization

1. Prepare representative live and dead cell samples. A dead-cell control can be generated using an established method appropriate for the cell type.
2. Using dead cells, determine an EthD-1 concentration that gives strong nuclear staining with minimal nonspecific cytoplasmic fluorescence. A practical starting range is 0.1–10 µM.
3. Using dead cells, test Calcein AM over approximately 0.1–10 µM and select a concentration that produces little fluorescence in dead cells.
4. Confirm the selected Calcein AM concentration on live cells and increase it only if the green signal is insufficient.
5. Use the resulting concentrations for the viability experiment, provided that clear separation of the populations is obtained.

Working Solution Preparation

A convenient starting formulation for many mammalian cell types is approximately 2 µM Calcein AM and 4 µM EthD-1 in D-PBS:

1. Add 20 µL of 2 mM EthD-1 stock to 10 mL D-PBS. Mix thoroughly to obtain approximately 4 µM EthD-1.
2. Add 5 µL of 4 mM Calcein AM stock to the EthD-1 solution and mix thoroughly. The resulting solution contains approximately 2 µM Calcein AM and 4 µM EthD-1.
3. Use the working solution immediately. The resulting DMSO concentration is approximately 0.1%.

Staining and Imaging

1. Add approximately 100–150 µL of the optimized staining solution to a 22 mm coverslip or sufficient volume to completely cover the cells.
2. Incubate for approximately 30–45 minutes at room temperature, protected from excessive light. Shorter incubations may be possible after optimization.
3. Transfer approximately 10 µL of fresh staining solution or D-PBS to a clean microscope slide.
4. Invert the coverslip onto the slide carefully and minimize evaporation during imaging.
5. Image the green and red channels separately or simultaneously using appropriate filter sets.

Fluorophore	Approx. excitation	Typical emission
Calcein	≈ 494 nm	≈ 517 nm
EthD-1	≈ 528 nm	≈ 617 nm; red/far-red detection

Fluorescence Microplate Protocol

Instrument Settings

For plate readers, acquire Calcein and EthD-1 fluorescence separately. A practical starting configuration is excitation near 485 ± 10 nm and emission near 530 ± 12.5 nm for Calcein, and excitation near 530 ± 12.5 nm with emission near 645 ± 20 nm for EthD-1. Instrument-specific filter sets may require optimization.

Cell Preparation

1. Culture adherent cells directly in the multiwell plate until the desired density is reached.
2. Wash adherent cells gently with approximately 500–1000 volumes of D-PBS and leave enough buffer to cover the well bottom.
3. Wash suspended cells similarly and collect them by centrifugation before transferring them to the plate.
4. As a starting point, use approximately 100 µL for a 250–300 µL flat-bottom well, approximately 70 µL for a 150–200 µL round-bottom well, or approximately 50 µL for a 100–150 µL conical well.
5. Apply cytotoxic treatments as required.
6. A practical working range is approximately 200–500 cells/well as a lower detection limit and up to about 10^6 cells/well as an upper starting point. The useful range is instrument-dependent.

Dye Concentration Optimization

1. Prepare live and dead control samples and select the appropriate plate-reader filters and sensitivity settings.
2. Determine the lowest EthD-1 concentration that provides near-maximal

fluorescence in dead cells; a starting range of 0.1–10 µM is appropriate.

3. Monitor the staining time course, for example at 10–15-minute intervals, when establishing a new assay.
4. Determine a Calcein AM concentration that gives negligible signal in dead cells but a clear signal in live cells; a starting range of 0.1–5 µM is useful.
5. Use the selected concentrations for subsequent experiments.

2× Working Solution Preparation

For a final concentration of 1 µM Calcein AM and 2 µM EthD-1 after 1:1 mixing with the cell suspension:

1. Add 20 µL of 2 mM EthD-1 stock to 10 mL D-PBS (≈4 µM EthD-1).
2. Add 5 µL of 4 mM Calcein AM stock and mix thoroughly (≈2 µM Calcein AM and 4 µM EthD-1).
3. The final DMSO concentration after 1:1 mixing with the cell suspension is approximately 0.1%.

Staining

1. Dispense 100 µL of the cell suspension into each well and add 100 µL of the 2× staining solution.
2. Incubate for the optimized period; 30–45 minutes at room temperature is a useful starting point.

Fluorescence Measurements and Controls

Use experimental samples together with single-color and live/dead controls. Keep cell number, reagent concentration, incubation time, temperature, and plate geometry constant across controls and experimental samples.

Sample	Channel	Purpose
Experimental cells + Calcein AM + EthD-1	Calcein and EthD-1	Measurement sample
All-dead cells + EthD-1	EthD-1	Maximum red reference
All-dead cells + Calcein AM	EthD-1	Minimum red reference/background
Live cells + EthD-1	Calcein	Minimum green reference
Live cells + Calcein AM	Calcein	Maximum green reference
Cell-free buffer ± dyes	Calcein and EthD-1	Instrument/reagent background

Interpretation and Calculation

After subtraction of appropriate cell-free background, the relative fraction of live and dead cells can be estimated from the calibrated fluorescence ranges:

% Live = [(F530,sample - F530,min) / (F530,max - F530,min)] × 100%

% Dead = [(F645,sample - F645,min) / (F645,max - F645,min)] × 100%

where, **F530,max** and **F530,min** are the green-channel values for live and EthD-1-only controls, respectively; **F645,max** and **F645,min** are the corresponding red-channel reference values from dead-cell/EthD-1 and dead-cell/Calcein controls. Background values measured in cell-free wells should be subtracted before calculation when necessary.

These equations are calibration-based estimates. For quantitative work, verify linearity and dynamic range for the specific plate reader, cell density, and assay conditions.

Determining Absolute Cell Numbers

1. Perform the viability measurement as described above.
2. After the measurement, lyse/kill the remaining cells using an established method, for example, approximately 0.1% saponin.
3. Mix the plate and allow the EthD-1 signal to equilibrate, typically for about

10 minutes.

4. Measure EthD-1 fluorescence at approximately 645 nm.
5. Generate a standard curve using known numbers of dead cells stained with a saturating EthD-1 concentration. Use the linear portion of the curve to estimate total cell number.

Flow Cytometry Protocol

1. Allow all reagents to reach room temperature.
2. Prepare a 50 µM Calcein AM working solution by diluting the 4 mM stock 1:80 in DMSO. Use the working solution within one day.
3. Prepare 1 mL of cell suspension at approximately $0.1–5\times10^6$ cells/mL.
4. Add 2 µL of 50 µM Calcein AM and 4 µL of 2 mM EthD-1 per 1 mL of cell suspension. Mix gently.
5. Incubate for 15–20 minutes at room temperature, protected from light.
6. Analyze as soon as practical, preferably within 1–2 hours after staining.
7. Use approximately 488 nm excitation. A practical starting configuration is 530/30 for Calcein and 610/20 for EthD-1.
8. Gate on cells to exclude debris and use single-color controls for standard fluorescence compensation.
9. Live cells should form a Calcein-positive/low-EthD-1 population, whereas membrane-compromised cells should form a Calcein-low/EthD-1-positive population.

Flow Cytometry with Absolute Counting Beads

1. Prepare and stain the cells as described in the flow-cytometry protocol.
2. Bring the counting-bead suspension to room temperature and vortex for approximately 30 s immediately before pipetting.
3. Add 50 µL of counting-bead suspension per 1 mL of stained sample and mix.
4. Acquire data promptly using 488 nm excitation and the same green/red fluorescence channels used for the viability assay.
5. Set the forward-scatter threshold low enough to retain the counting beads. Gate cells separately from beads and exclude debris.
6. Collect at least 1,000 bead events for a statistically useful estimate of sample volume.
7. If beads cannot be resolved in the chosen fluorescence parameters, use another scatter/fluorescence parameter combination for the bead gate.
8. Absolute cell concentration calculation:

   Cell concentration (cells/µL) = (A × C) / (B × D)

   where, **A** is a number of cell events; **B** is a number of bead events; **C** is a assigned bead count in the bead aliquot used (beads per aliquot); **D** is a sample volume represented by the analyzed cell suspension (µL).

   Example: if 1,700 cell events and 1,030 bead events are collected from a 1,000 µL sample and the bead lot contains 49,500 beads per 50 µL aliquot, the estimated concentration is approximately 81.7 cells/µL.
9. Correct the calculation for any sample dilution or for a different volume of counting-bead suspension.

22.09.509-QM
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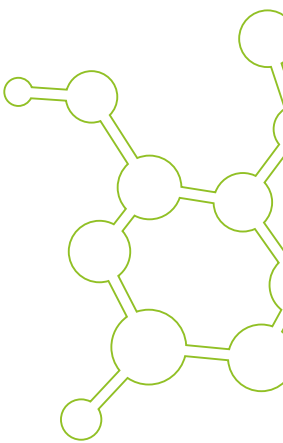

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