PeptiMatrix™
Next generation peptide hydrogels

STANDARD OPERATING PROCEDURE

LIVE/DEAD STAINING FOR CELL VIABILITY OF CELLS IN PEPTIMATRIX™ HYDROGELS

1. BACKGROUND

The LIVE/DEAD kit is a two-colour fluorescence cell viability assay. Calcein AM, which is enzymatically converted to the highly fluorescent calcein by intracellular esterase activity, can be used to detect live cells since it is cell-permanent and well retained within live cells. Ethidium homodimer, which is excluded by the intact plasma membrane of live cells, enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence on binding to nucleic acids, producing a bright red fluorescence in dead cells. Background fluorescence levels are inherently low with this technique as the dyes are virtually non-fluorescent before interacting with the cells. This assay technique is widely used in fluorescence microscopy and flow cytometry, and is fast and easy to carry out on cells encapsulated in PeptiMatrix™ hydrogels.

2. RISK ASSESSMENT

Always follow your organisation's laboratory safety procedures.

Work inside an appropriate microbiology safety cabinet for your cell type. Refer to the **PeptiMatrix Safety Data Sheet (SDS)** for detailed safety, handling, storage, and first aid information relating to the hydrogel components.

If you are working with additional cell lines, media supplements, matrix additives, or other reagents, consult the relevant SDS documents for those materials as well.

3. MATERIALS

- Class II microbiology safety cabinet (or appropriate class for your cell type)
- Cells encapsulated or seeded on PeptiMatrix hydrogels, plated in appropriate microplate e.g., black-bordered well plate with clear optical bottom (e.g. ThermoFisher #165305 - Nunc™ 96-Well Optical-Bottom Microplate, black, TC surface)

- LIVE/DEAD kit containing Calcein AM and Ethidium homodimer (e.g. Fisher #L3224 - LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells)
- P1000, P200 and P10 pipettes and filter tips
- Dulbecco's Phosphate Buffered Saline (DPBS)
- Foil
- 1.5mL microcentrifuge tubes
- Fluorescence microscope

Optional materials:

- Scalpel blade (optional for hydrogels in transwells)
- Glass coverslips or imaging dishes (optional for hydrogels in transwells)

4. METHODS

4.1 Preparing the LIVE/DEAD reagent

This procedure can be scaled up or down depending on the number of wells you plan to prepare, and which well plate size you are using.

For reference, the thawing and dilution steps described below are based on the Fisher #L3224 - LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells, comprised of **4 mM Calcein AM** and **2 mM Ethidium homodimer** stock concentrations, and should be adjusted accordingly depending on the kit used.

1. Remove reagents from -20 °C and allow to warm to room temperature (protected from light).
2. Centrifuge vials briefly before opening.
3. Prepare aqueous working solution containing Calcein AM and Ethidium homodimer immediately prior to use.
    - Prepare the same volume of working solution per well as the volume of hydrogel in the well e.g. for a 96 well plate with 100 µL hydrogel per well, prepare 100 µL LIVE/DEAD working solution per well. Include 10% extra volume to account for pipetting errors.
    - Using a P10 or P2 pipette, add 0.5 µL Calcein AM and 2 µL Ethidium homodimer per 1 mL DPBS. This working solution will have a final concentration of 2 µM Calcein AM and 4 µM Ethidium homodimer.
    - Ensure the working solution is well mixed and cover in foil to protect from light.

4.2 LIVE/DEAD staining cells encapsulated in PeptiMatrix hydrogels

This procedure can be scaled up or down depending on the number of wells you plan to prepare, and which well plate size you are using.

For reference, the steps below describe the process for cells encapsulated in 100 µL hydrogel per well plated into a 96-well plate.

1. Using a P200 pipette, aspirate the old media on top of the gel from the well, removing ~75% of the media volume to minimise risk of disturbing the gel.
2. Wash cells gently in DPBS to remove any serum esterase activity present in serum containing media. These esterases can hydrolyse Calcein AM to cause an increase in extracellular fluorescence. Add 100 µL DPBS per well, then aspirate the DPBS as described in Step 1 above.
3. Using a P200 pipette, add 100 µL working LIVE/DEAD solution per well on top of the gel, taking care not to disturb it.
4. Cover the plate with foil and incubate in the dark at room temperature for 10-15 minutes.
5. Using a P200 pipette, aspirate the working solution on top of the gel from the well as described in Step 1.
6. Add 100 µL DPBS per well to the gels, then use for downstream applications such as plate reading or fluorescence microscopy.

4.3 LIVE/DEAD staining cells encapsulated in PeptiMatrix hydrogels in transwells

This procedure can be scaled up or down depending on the number of wells you plan to prepare, and which well plate/transwell size you are using.

For reference, the steps below describe the process for cells encapsulated in 200 µL hydrogel plated into transwell inserts suitable for 24 well plates.

1. Using a P200 pipette, aspirate the old media on top of the gel from the well, removing ~75% of the media volume to minimise risk of disturbing the gel.
2. Wash cells gently in DPBS to remove any serum esterase activity present in serum containing media. These esterases can hydrolyse Calcein AM to cause an increase in extracellular fluorescence. Add 200 µL DPBS per well, then aspirate the DPBS as described in Step 1 above.
3. Using a P200 pipette, add 200 µL working LIVE/DEAD solution per well on top of the gel, taking care not to disturb it.
4. Cover the plate with foil and incubate in the dark at room temperature for 10-15 minutes.
5. Using a P200 pipette, aspirate the working solution on top of the gel from the well as described in Step 1.
6. Remove the transwell from the 24 well plate using sterile tweezers and remove the membrane from the bottom of the transwell using a scalpel. Run the scalpel around the edge of the transwell and remove the membrane using tweezers.
7. Place the transwell on a coverslip or imaging dish and allow the hydrogel to attach to the glass/plastic. Slide the transwell up carefully, leaving behind the hydrogel attached to the

surface. Alternatively, gently push the gel out of the transwell using the wide end of a filter tip.

8. Use for downstream applications such as fluorescence microscopy.

4.3 Fluorescence microscopy of stained cells

For reference, the steps below describe the process for cells encapsulated in 100 µL hydrogel per well plated into a 96-well plate.

1. Prepare samples of experimental cells and stain each well with LIVE/DEAD stain as described in Methods section 4.2.
2. Ensure the stained hydrogels are imaged the same day as staining, as Calcein AM is susceptible to hydrolysis.
3. View labelled cells under a fluorescence microscope with appropriate optical filters. Calcein AM and Ethidium homodimer can be viewed simultaneously with a conventional fluorescein longpass filter. However, the fluorescence from these dyes can also be observed separately; Calcein AM with a standard fluorescein bandpass filter and Ethidium homodimer with filters for propidium iodide or Texas Red® dye. The fluorescence emissions should be acquired separately, Calcein AM at **530** ± 12.5 nm, and Ethidium homodimer at **645** ± 20 nm.

5. DISPOSAL

Dispose of hydrogels containing cells, media, or matrix components according to your local guidelines for biological waste.

Dispose of Calcein AM and Ethidium homodimer waste according to your institute guidelines.

6. DOCUMENT HISTORY

Version	Date	Summary of Changes
1.0	**05 Feb 26**	First version of customer facing SOP, adapted from internal PeptiMatrix LIVE/DEAD procedures.