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Protocol for Loading Cal Red™ R525/650 AM Into Live Cells

IMPORTANT DISCLAIMER: The following is a sample protocol for loading Cal Red™ R525/650 AM esters into live cells. This protocol only provides a guideline and should be modified according to your experimental needs. Please read the entire protocol before starting.

How to use this protocol:

First, select your microplate format and enter in the required specifications. Next, follow the instructions provided in each section to prepare the necessary buffers, stock solutions, and working solutions needed to successfully load your cells with Cal-520®, AM. For assistance, use tools and calculators to determine the amount of component required for each part of the loading procedure.

Key parameters

Instrument:	Fluorescence microplate reader
Excitation	490
Emission	525, 660
Cutoff	515, 630
Recommended plate	Solid black

Instrument:	Fluorescence microscope
Excitation	FITC
Emission	FITC
Recommended plate	Black wall/clear bottom

Instrument:	Flow cytometer
Excitation	Ratio: FITC/PerCP
Emission	Ratio: FITC/PerCP

Select your microplate format

Black wall/clear bottom microplate:	96-wells
Enter the number of wells to be used:	32
Volume of culture medium per well ¹ :	100 μ L
Volume of working solution per well ¹ :	100 μ L

Prepare these materials

IMPORTANT NOTE: This protocol includes the non-ionic detergent Pluronic® F-127 and the organic anion-transport inhibitor probenecid. Both reagents are not required, but highly recommended. To remove a reagent from the loading protocol, uncheck the appropriate box:

Required

- ☒ Cal Red™ R525/650 AM
- ☒ Hanks and Hepes Buffer *(HHBS) or a buffer of your choice
- ☒ 100% DMSO

Optional

- ☒ 10% Pluronic® F-127
- ☒ 25 mM Probenecid

Step-by-step guide:

1. Prepare an HHBS buffer, a 10% Pluronic® F-127 solution, and a 25 mM Probenecid solution.

- a. For instructions on how to prepare a HHBS buffer, see our buffer recipe page
- b. For instructions on how to prepare a 10% Pluronic® F-127 solution, see recipe
- c. For instructions on how to prepare a 25 mM Probenecid solution, see recipe

2. Prepare a 2 mM to 5 mM Cal Red™ R525/650 AM stock solution in high quality anhydrous DMSO.

- a. Amount of Cal Red™ R525/650 AM to use: 1 mg
- b. Desired concentration: 2 mM
- c. In a suitable container mix **1 mg** of Cal Red™ R525/650 AM with **-454.13 μ L** of anhydrous DMSO.

3. Prepare a 2X working solution in HHBS with 10 μ M Cal Red™ R525/650 AM⁴, 0.08% Pluronic® F-127 and 2 mM Probenecid.

- a. Final in-well concentration of Cal Red™ R525/650 AM: 5 μ M
- b. Final in-well concentration of Pluronic® F-127: 0.04 %
- c. Final in-well concentration of Probenecid: 1 mM
- d. In a suitable container mix **16 μ L** of Cal Red™ R525/650 AM, **25.6 μ L** of 10% Pluronic® F-127, and **256 μ L** of 25 mM Probenecid. Next, add HHBS or a buffer of your choice until the volume is **3.2 mL**.

Note: For most cell lines we recommend the final concentration of Cal Red™ R525/650 AM be 4 to 5 μ M.

Note: Recommended final in well concentration of Pluronic F-127 is 0.02% to 0.04%.

Note: Recommended final in well concentration of Probenecid is 1 to 2.5 mM.

4. Add **100 μ L** of the dye working solution into the desired wells already containing **100 μ L** of culture medium.

- a. This step will dilute the dye working solution from 2X to 1X and adjust the final concentrations of each component to the following: **5 μ M** of Cal Red™ R525/650 AM, **0.04%** Pluronic® F-127, **1 mM** Probenecid.

5. Incubate the dye-loading plate⁵.

- a. Incubate the dye-loading plate in a cell incubator for 20-120 minutes.
- b. Incubate the dye-loading plate at room temperature for 30 minutes.

6. Prepare an HHBS buffer (or a buffer of your choice) with 1.0 mM Probenecid.

- a. In a suitable container add 160 µL of 25 mM Probenecid. Next, add HHBS or a buffer of your choice until the volume is 4 mL.

7. Replace the dye working solution with the HHBS buffer or a buffer of your choice with 1.0 mM Probenecid.

- a. First, remove 200 µL of the dye working solution and culture medium from the desired wells.
- b. To those same wells add back 200 µL of HHBS (or a buffer of your choice) with 1.0 mM Probenecid.

8. Run your assay.

- a. Add desired treatment to your sample.
- b. Run the experiment as Ex/Em = 492/650 nm.

Additional Information:

Cal Red™ R525/650 AM Specifications

Excitation:	492
Emission:	650
Molecular Weight:	~1100
Solvent:	DMSO
Extinction Coefficient:	N/A
K _d :	330 nM

1 M NaOH Recipe

1. Prepare 2 mL of distilled water in a suitable container.
2. Slowly add 100 mg of NaOH to the solution with mixing. *
3. Add distilled water until volume is 2.5 mL.
4. Store solution in plastic container at room temperature.

**This is an exothermic process, proper precautions and guidelines should be followed.*

10% Pluronic F-127 Recipe:

1. Dissolve 1 g of Pluronic® F-127 (Cat# 20050) in 10 mL of distilled water to make a 10% (w/v) stock solution.
2. Heat 10% Pluronic® F-127 stock solution for about 30 minutes at a temperature ranging from 40 to 50 °C.
3. Store excess 10% Pluronic® F-127 according to its storage specifications.

25 mM Probenecid Recipe:

1. In a suitable container, dissolve 1 vial (72 mg) of Probenecid (Cat# 20060) in 0.3 mL of 1 M NaOH.
2. Add HHBS or a buffer of your choice until the volume is 10 mL.
3. Aliquot and store any unused 25 mM Probenecid solution according to its storage specifications.

Storage Conditions

- It is recommended to prepare and use Cal Red™ R525/650 AM stock solution on the same day. However, if stock solutions need to be prepared in advance we recommend storing the Cal Red™ R525/650 AM stock solution as aliquots in tightly sealed vials at -20°C, desiccated and protected from light. Under these conditions, AM esters should be stable for 3 months.
- 10% Pluronic F-127 stock solution must be stored at room temperature (DO NOT FREEZE) for up to 6 months.
- 20 mM Probenecid *stabilized in aqueous solution* may be stored at -20°C and protected from light for up to 6 months. Avoid repeated freeze-thaw cycles.

Notes

1. Volumes can be adjusted according to the need and volume of the experiment setups.
 2. Pluronic® F-127 (PF-127) is a nonionic surfactant and relatively non-toxic to cells. PF-127 is commonly used with dye AM esters to improve their aqueous solubility.
 3. If your cells contain organic anion-transporters, Probenecid (0.5-1.0 mM) may be added to the dye working solution to reduce the leakage of the de-esterified indicators.
 4. The exact concentration of the indicator required for cell loading must be determined empirically.
- SAMPLE