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Protocol for Loading Fluo-5N, AM *Cell permeant* Into Live Cells

IMPORTANT DISCLAIMER: The following is a sample protocol for loading Fluo-5N, AM *Cell permeant* 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 Fluo-5N, AM *Cell permeant*. 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
Cutoff	515
Recommended plate	Solid black

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

Instrument:	Flow cytometer
Excitation	488 nm laser
Emission	530/30 nm filter
Instrument specification(s)	FITC channel

Select your microplate format

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

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

☑ Fluo-5N, AM *Cell permeant*

☑ 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 Fluo-5N, AM *Cell permeant* stock solution in high quality anhydrous DMSO.

a. Amount of Fluo-5N, AM *Cell permeant* to use: 1 mg
b. Desired concentration: 2 mM
c. In a suitable container mix 1 mg of Fluo-5N, AM *Cell permeant* with 443.29 µL of anhydrous DMSO.

3. Prepare a 2X working solution in HHBS with 10 µM Fluo-5N, AM *Cell permeant* [4], 0.08% Pluronic® F-127 and 2 mM Probenecid.

a. Final in-well concentration of Fluo-5N, AM *Cell permeant*: 5 µM
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 µL of Fluo-5N, AM *Cell permeant*, 25.6 µL of 10% Pluronic® F-127, and 256 µL 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 Fluo-5N, AM *Cell permeant* be 2 to 5 µM.*
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 µL of the dye working solution into the desired wells already containing 100 µL 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 µM of Fluo-5N, AM *Cell permeant*, 0.04% Pluronic® F-127, 1 mM Probenecid.

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5. Incubate the dye-loading plate[5].
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 = 494/516 nm.

Additional Information:

Fluo-5N, AM *Cell permeant* Specifications

Excitation:	494
Emission:	516
Molecular Weight:	1127.92
Solvent:	DMSO
Extinction Coefficient:	N/A
K_d:	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.

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Storage Conditions

- It is recommened to prepare and use Fluo-5N, AM *Cell permeant* stock solution on the same day. However, if stock solutions need to be prepared in advanced we recommend storing the Fluo-5N, AM *Cell permeant* stock solution as aliquots in tightly sealed vials at -20°C, dessicated 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.