Fluo-4 Direct™ Calcium Assay Kits

Catalog nos. F10471, F10472, F10473

Table 1. Contents and storage information.

Material	Amount			Storage*	Stability
	F10471	F10472	F10473		
Fluo-4 Direct™ calcium assay reagent (Component A)	10 × 10 mL	1 × 100 mL	1 × 1 L	• ≤–20°C • Desiccate • Protect from light	When stored as directed the kit is stable for at least 6 months.
Probenecid, water-soluble (Component B)	2 × 77 mg	2 × 77 mg	1 × 1.54 g		
Fluo-4 Direct™ calcium assay buffer (Component C)	200 mL	200 mL	Not supplied		
*These storage conditions are appropriate when storing the entire kit upon receipt. For optimal storage conditions for each component, refer to the vial labels.					
Number of assays: Sufficient material is supplied for 20 × 96- or 384-well plates (Starter pack, Cat. no. F10471) or 20 × 96- or 384-well plates in a 1 × 100 mL (Surveyor pack, Cat. no. F10472) or 200 × 96- or 384-well plates in a 1 × 1 L (High-throughput pack, Cat. no. F10473) based on the protocol below.					
Approximate fluorescence excitation/emission maxima: Fluo-4 Direct™ calcium reagent: 495/516 in nm.					

Introduction

Fluo-4 Direct™ is an advanced fluorescent Ca^{2+} indicator that complements the Invitrogen portfolio by addressing a key limitation in assay measurement; background fluorescence. Fluorescent Ca^{2+} indicators are widely used for in-cell measurement of agonist-stimulated and antagonist-inhibited calcium signaling through G protein-coupled receptors (GPCRs), a large and active target class in drug discovery, in addition to the measurement of ligand and voltage-gated ion channel activity. The visible excitation wavelength, high sensitivity, and large fluorescence increase upon binding Ca^{2+} has made them the indicators of choice for calcium detection. Homogeneous cell-based assays for calcium have gained popularity based on the promise of fewer steps, lower variability, and easier protocols. These characteristics allow for automation of many steps and consequently, lend themselves to multi-well microplate based assays important for high-throughput capability. However, many of the current fluorescent Ca^{2+} indicators require media removal and washing steps to achieve good results.

The Fluo-4 Direct™ Calcium Assay Kits offer a proprietary assay formulation that allows direct addition to wells containing cells growing in culture media without the requirement of media removal or a wash step. Eliminating the media removal step from the workflow can result in lower variability and higher Z´ values compared to the standard fluo-4 assay, while providing an easier and faster assay. Contributions to baseline fluorescence by the growth medium are eliminated by the addition of a suppression dye which reduces background fluorescence.

Another source of potential fluorescence outside the cells is extrusion of the indicator out of the cell by organic anion transporters. A proprietary, water-soluble Probenecid is supplied with the Fluo-4 Direct™ Calcium Assay Kits, which is commonly used to inhibit this transport and reduce the baseline signal. The water-soluble form of probenecid is easy to dissolve in buffer and safer to use than the free acid form, which requires caustic 1 M NaOH to dissolve. The Fluo-4 Direct™ Calcium Assay Kits are designed for microplates and high-throughput screening (HTS), and the assay can be performed on adherent as well as non-adherent cells. The Fluo-4 Direct™ Calcium Assay Kit, Starter pack (Cat. no. F10471) and Surveyor pack (Cat. no. F10472) contains sufficient reagents for 20 microplates and includes Fluo-4 Direct™ calcium assay buffer (Component C). The Fluo-4 Direct™ Calcium Assay Kit, High-throughput pack (Cat. no. F10473) contains sufficient reagents for 200 microplates and does not include the Fluo-4 Direct™ calcium assay buffer (Component C).

Before Starting

Materials Required but Not Provided

- Cells and culture media
- Poly-D-Lysine coated 96- or 384-well microplates

For high-throughput kit only:

- 1X Hanks' balanced salt solution (HBSS), 1.5 L (Invitrogen Cat. no. 14025-092)
- 1 M HEPES buffer solution, 30 mL (Invitrogen Cat. no. 15630-106)

Caution

Probenecid (Component B) is harmful if swallowed. Wear appropriate protective laboratory clothing, gloves, and safety glasses when handling this reagent.

General Recommendations

- Prepare reagents on the day that you perform your experiments. Ensure that Components A and B are completely dissolved when making the reagent solutions.
- Reagent quantities are given for assaying two (Starter pack), twenty (Surveyor pack), or two hundred microplates at a time (high-throughput pack); scale up quantities according to your experiment.
- When following the directions below, the Fluo-4 Direct™ calcium assay reagent solution is at a 2X concentration. If you plan to remove media and add the Fluo-4 Direct™ calcium assay reagent directly to cells, dilute the Fluo-4 Direct™ calcium assay reagent solution to 1X with media or assay buffer. For example, if using the Starter Pack (Cat. no. F10471), you need to add 10 mL of additional buffer per bottle to bring the reagent concentration to 1X before adding the reagent to the wells. Additionally, if you are going to use the Fluo-4 Direct™ calcium reagent loading solution at 1X, there is no need to add additional probenecid which can be toxic to the cells as the final probenecid concentration will be 2.5 mM.
- Fluo-4 Direct™ calcium assay reagent solution made from Component A can be frozen for up to 7 days at –20°C and reused with good results.

Experimental Protocol for Adherent Cells

Preparing Cells

Culture adherent cells in 96- or 384-well microplates (poly-D-Lysine–coated plates recommended), to near confluence. The following assay was developed with M1WT3 (CHO

M1) cell line (ATCC Cat. no. CRL-1985™), GeneBLAzer® M1-NFAT-*bla* Jurkat (Invitrogen Cat. no. K1710), GeneBLAzer® H1-NFAT-*bla* HEK293T (Invitrogen Cat. no. K1703) and U-2 OS (ATCC Cat. no. HTB-96™) cells with BacMam expressed muscarinic 1 and muscarinic 3 GPCRs.

In 384-well plates, CHO, U-2 OS, and HEK293 cells can be plated at 5–10,000 cells per well and grown overnight. If you are using 96-well plates, plate 4–8 times these cell numbers per well. Alternatively, cells can be plated at higher densities and used after a 4–6 hour incubation. The success of this approach is cell-type dependent.

Preparing Reagents

If you are using the Starter pack (Cat. no. F10471) or Surveyor pack (Cat. no. F10472), the Fluo-4 Direct™ calcium assay buffer (Component C) is supplied with the kit, proceed directly to step 1.2.

The High-throughput pack (Cat. no. F10473) does not include Fluo-4 Direct™ calcium assay buffer (Component C), proceed to step 1.1 to prepare the buffer.

1.1 For High-throughput pack, make the quantity of Fluo-4 Direct™ calcium assay buffer you need, based on the number of microplates in your experiment. For 200 microplates, a total of 1.5 L of assay buffer is sufficient for all reagent preparations. Prepare 1.5 L of Fluo-4 Direct™ calcium assay buffer by adding 30 mL of 1 M HEPES to 1.47 L of 1X HBSS. Adjust the pH of the buffer to 7.3 with NaOH.

1.2 Prepare 250 mM stock solution of probenecid by adding 1 mL of Fluo-4 Direct™ calcium assay buffer to each 77 mg vial of water-soluble probenecid (Component B for Cat. nos. F10471 and F10472) or 20 mL of Fluo-4 Direct™ calcium assay buffer to the 1.54 g bottle of water soluble probenecid (Component B for Cat. No. F10473). Vortex until dissolved. Store any unused probenecid stock solution at ≤–20°C for up to 6 months.

Note: Water-soluble probenecid is supplied but is optional for the assay. For best results with Fluo-4 Direct™, you may need to use probenecid for certain cell types, while it may not be necessary for other cell types.

1.3 Prepare the 2X Fluo-4 Direct™ calcium reagent loading solution with a final probenecid concentration of 5 mM for the kit that you are using, as follows:

Starter pack (Cat. no. F10471): Add 10 mL Fluo-4 Direct™ calcium assay buffer and 200 µL 250 mM probenecid stock solution to one bottle of Fluo-4 Direct™ calcium reagent (Component A). This 2X Fluo-4 Direct™ calcium reagent loading solution is sufficient for two microplates.

Surveyor pack (Cat. no. F10472): Add 100 mL of Fluo-4 Direct™ calcium assay buffer and 2 mL 250 mM probenecid stock solution to one bottle Fluo-4 Direct™ calcium reagent (Component A). This 2X Fluo-4 Direct™ calcium reagent loading solution is sufficient for 20 microplates.

High-throughput pack (Cat. no. F10473): Add 1 L Fluo-4 Direct™ calcium assay buffer and 20 mL 250 mM probenecid stock solution to one bottle of Fluo-4 Direct™ calcium reagent (Component A). This 2X Fluo-4 Direct™ calcium reagent loading solution is sufficient for 200 microplates.

Note: Vortex and allow the solution to sit for 5 minutes to ensure that the reagent is completely dissolved and then vortex again. Ensure that reagent is completely dissolved before proceeding with loading cells.

1.4 If needed, prepare solutions of the receptor ligands for your experiment in Fluo-4 Direct™ calcium assay buffer (without probenecid).

2.1 Remove microplates containing cells from the incubator. Add an equal volume of 2X Fluo-4 Direct™ calcium reagent loading solution (from step 1.3) directly to wells containing cells in culture medium.

For example: Add 50 µL of the 2X Fluo-4 Direct™ calcium reagent loading solution per well of a 96-well plate containing 50 µL of growth medium per well or 12.5 µL of the 2X Fluo-4 Direct™ calcium reagent loading solution per well of a 384-well plate containing 12.5 µL of growth medium per well.

2.2 Incubate plates at 37°C for 30–60 minutes. You can perform incubations for a period of time at 37°C and at room temperature. However, the total loading time (incubation time) should be at least 60 minutes.

Note: We obtained best results with an incubation of 30 minutes at 37°C and 30 minutes at room temperature for CHO M1 and U-2 OS cells.

The plates are now ready for use in an experiment without removing the medium or Fluo-4 Direct™ calcium reagent loading solution from the wells. The amount of time the assay can be successfully run after loading is cell-type dependent. We obtained good results from CHO-M1 cells kept at room temperature for up to 5 hours after loading with Fluo-4 Direct™ reagent.

2.3 Measure fluorescence using instrument settings appropriate for excitation at 494 nm and emission at 516 nm.

Experimental Protocol for Non-adherent Cells

Preparing Cells

Prior to pelleting cells (step 3.3), ensure that you have sufficient Fluo-4 Direct™ calcium assay buffer for the experiments.

If you are using the Starter pack (Cat. no. F10471) or the Surveyor pack (Cat. no. F10472), the Fluo-4 Direct™ calcium assay buffer (Component C) is supplied with the kits, proceed directly to step 3.2.

The High-throughput pack (Cat. no. F10473) does not include Fluo-4 Direct™ calcium assay buffer (Component C), proceed to step 3.1 to prepare the buffer.

3.1 For the High-throughput pack, make the quantity of Fluo-4 Direct™ calcium assay buffer you need, based on the number of microplates in your experiment. For 200 microplates, a total of 1.5 L of assay buffer is sufficient for all reagent preparations. Prepare 1.5 L of Fluo-4 Direct™ calcium assay buffer by adding 30 mL of 1 M HEPES to 1.47 L of 1X HBSS. Adjust the pH of the buffer to 7.3 with NaOH.

3.2 Count the density of cells directly from the cell culture flask. Calculate the amount of cells needed for plating so that they cover the bottom of the well when settled.

For Jurkat cell line (GeneBLAzer® muscarinic receptor (M1) and NFAT-*bla* Jurkat cell line, (Invitrogen Cat. no. K1051), 125,000 cells per well in a 96-well plate, or 31,250 cells per well in a 384-well plate performed well in the assay.

For example, if the cell culture density is 1.5×10^6 cells/mL, then for each microplate (~100 wells in 96-well plates or ~400 wells in 384-well plates) you need:

125,000 cells × 100 (or 31,250 cells × 400) = 1.25×10^7 cells

1.25×10^7 cells = 8.3 mL of 1.5×10^6 cells/mL cell culture required

3.3 Pellet cells from the required amount of the culture by centrifuging at ~200 × g (1,000 rpm) for 3 minutes.

3.4 Remove the medium and resuspend the cell pellet in Fluo-4 Direct™ calcium assay buffer to a density of ~2.5×10^6 cells/mL (125,000 cells/50 µL in 96-well plates, or 31,250 cells/12.5 µL in 384-well plates). The volume of Fluo-4 Direct™ calcium assay buffer needed to resuspend the cells can be obtained by multiplying 50 µL or 12.5 µL by the number of wells that you need.

For example:
50 µL per well × 100 wells in 96-well plates = 5 mL
12.5 µL per well × 400 wells in 384-well plates = 5 mL

3.5 Pipet the resuspended cells, 50 µL per well or 12.5 µL per well, into the microplate(s). If desired, pipet the same volume of the assay buffer alone into no-cell control wells.

3.6 Incubate the plate(s) at 37°C and 5% CO_2 for 60 minutes to allow the cells to settle.

Preparing Reagents

4.1 Prepare 250 mM stock solution of water-soluble probenecid by adding 1 mL Fluo-4 Direct™ calcium assay buffer to each 77 mg vial of probenecid (Component B for Cat. nos. F10471 and F10472) or 20 mL of Fluo-4 Direct™ calcium assay buffer to the 1.54 g bottle of probenecid (Component B for Cat. no. F10473). Vortex until dissolved. Store any unused probenecid stock solution at ≤–20°C for up to 6 months.

4.2 Prepare a 2X Fluo-4 Direct™ calcium reagent loading solution with a 5 mM final probenecid concentration for the kit that you are using, as follows:

Starter pack (Cat. no. F10471): Add 10 mL Fluo-4 Direct™ calcium assay buffer and 200 µL 250 mM probenecid stock solution to one bottle of Fluo-4 Direct™ calcium reagent (Component A). This 2X Fluo-4 Direct™ calcium reagent loading solution is sufficient for two microplates.

Surveyor pack (Cat. no. F10472): Add 100 mL of Fluo-4 Direct™ calcium assay buffer and 2 mL 250 mM probenecid stock solution to one bottle Fluo-4 Direct™ calcium reagent (Component A). This 2X Fluo-4 Direct™ calcium reagent loading solution is sufficient for 20 microplates.

High-throughput pack (Cat. no. F10473): Add 1 L Fluo-4 Direct™ calcium assay buffer and 20 mL 250 mM probenecid stock solution to one bottle of Fluo-4 Direct™ calcium reagent (Component A). This 2X Fluo-4 Direct™ calcium reagent loading solution is sufficient for 200 microplates.

Note: Vortex and allow the solution to sit for 5 minutes to ensure that the reagent is completely dissolved and then vortex again. Ensure that reagent is completely dissolved before proceeding with loading cells.

4.3 If needed, prepare solutions of the receptor ligands for your experiment in Fluo-4 Direct™ calcium assay buffer (without probenecid).

Fluo-4 Direct™ Calcium Assay

5.1 Remove plate(s) containing the cells from the incubator and add an equal volume of 2X Fluo-4 Direct™ calcium reagent loading solution (from step 4.2) directly into wells containing cells

in culture media.

For example, add 50 µL of the 2X Fluo-4 Direct™ calcium reagent loading solution per well of a 96-well plate containing 50 µL of growth medium per well or 12.5 µL of the 2X Fluo-4 Direct™ calcium reagent loading solution per well of a 384-well plate containing 12.5 µL of growth medium per well.

5.2 Incubate plates at 37°C for 30–60 minutes. You can perform incubations for a period of time at 37°C and at room temperature. However, the total loading time (incubation time) should be at least 60 minutes.

Note: We obtained best results with an incubation of 30 minutes at 37°C and 30 minutes at room temperature for CHO M1 and U-2 OS cells.

The plates are now ready for use in an experiment without the need to remove the medium or Fluo-4 Direct™ calcium reagent loading solution from the wells. The amount of time the assay can be successfully run after loading is cell-type dependent. We obtained good results from CHO-M1 cells kept at room temperature for up to 5 hours after loading with Fluo-4 Direct™ reagent.

5.3 Measure fluorescence using instrument settings appropriate for excitation at 494 nm and emission at 516 nm.

Typical Results

CHO M1 cells were treated with agonists and antagonists, and assayed for calcium response using Fluo-4 Direct™ Calcium Assay Kit. Responses agreed with published results as shown in Figure 1.

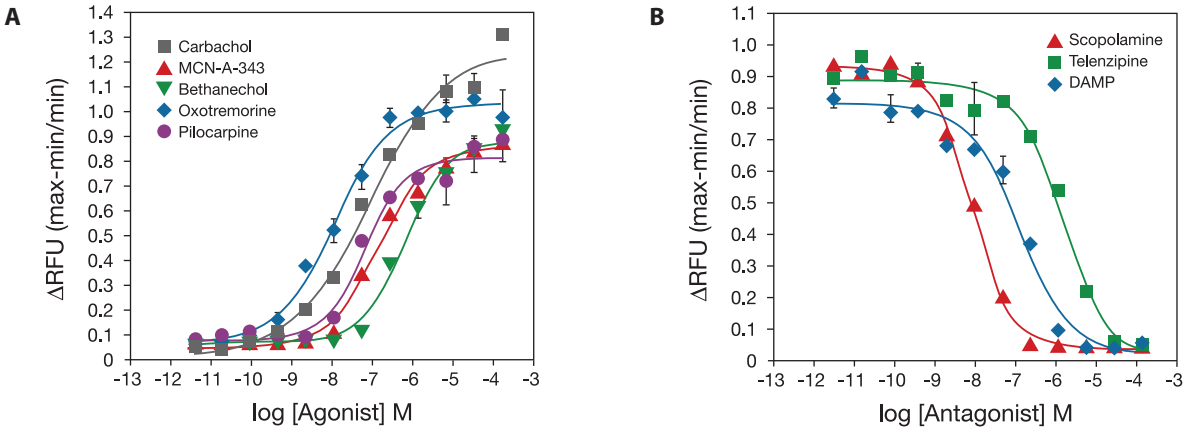

Figure 1. Dose-dependent calcium response to muscarnic 1 (M1) receptor agonists and antagonists. CHO M1 cells were plated in a poly-D-Lysine coated 384-well plate and incubated overnight. The following day, cells were assayed for a calcium response to carbachol using the Fluo-4 Direct™ Calcium Assay Kit. Cells were stimulated with agonists, carbachol, MCN-A-343, bethanechol, oxotremorine, and pilocarpine (panel A) or cells were treated with antagonists, scopolamine, telenzipine, and DAMP to block the calcium response elicited by 114 nM carbachol (EC_{80} for this receptor) (panel B). Measurements are given in relative fluorescent units (RFU) as the maximum response minus the minimum response divided by the minimum response. Rank order of agonist and antagonist potency agreed with published results.

Product List Current prices may be obtained from our website or from our Customer Service Department.

Cat. no.	Product Name	Unit Size
F10471	Fluo-4 Direct™ Calcium Assay Kit, Starter pack	1 kit
F10472	Fluo-4 Direct™ Calcium Assay Kit, Surveyor pack	1 kit
F10473	Fluo-4 Direct™ Calcium Assay Kit, High-throughput pack	1 kit
Related Products		
P10020	PowerLoad™ concentrate, 100X	5 mL
P36400	Probenecid, water soluble	10 × 77 mg
14025-092	Hanks' Balanced Salt Solution (HBSS) (1X), liquid	500 mL
15630-106	HEPES Buffer Solution (1 M)	20 mL

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