

## Fluo-4 NW Calcium Assay Kits (F36205, F36206)

**F36205 Fluo-4 NW Calcium Assay Kit (high-throughput) \*for 100 microplates\***

**F36206 Fluo-4 NW Calcium Assay Kit (starter pack with buffer) \*for 10 microplates\***

### Quick Facts

#### Storage upon receipt:

- Component A: Store at  $\leq -20^{\circ}\text{C}$ ; desiccate and protect from light
- Component B: Store at  $\leq 25^{\circ}\text{C}$ ; desiccate
- Component C (included in starter pack only):  
For periods of  $\leq 2$  weeks, store at  $\leq 6^{\circ}\text{C}$ ; protect from light. For long-term storage, store at  $\leq -20^{\circ}\text{C}$ ; protect from light.

**Ex/Em:** 494/516 nm

### Introduction

Fluo-4 AM is a fluorescent  $\text{Ca}^{2+}$  indicator that is widely used for in-cell measurement of agonist-stimulated and antagonist-inhibited calcium signaling in high-throughput screening (HTS) applications. Its visible wavelength excitation (compatible with argon-ion laser sources), high sensitivity, and large fluorescence increase upon binding  $\text{Ca}^{2+}$  has made it the indicator of choice for characterizing G-protein-coupled receptor (GPCR) pharmacology and function.<sup>1</sup> These properties have made fluo-4 AM attractive not only for microplate screening applications but for microscopy and flow cytometry as well.

Homogeneous cell-based assays for calcium have gained popularity based on the promise of fewer steps, lower variability, and easier protocols for non-adherent cell lines. However, most of these assays incorporate the use of a quencher dye, which can interact negatively with some receptor systems.<sup>2</sup>

Molecular Probes' Fluo-4 NW (No-Wash) Calcium Assay Kits offer a proprietary assay formulation that requires neither a wash step nor a quencher dye. The fluo-4 NW assay achieves larger increases in fluorescence intensity than standard fluo-3 and fluo-4 assays with a wash step, and Molecular Devices' Calcium 3 assay, which uses a quencher dye. Eliminating the wash step results in lower variability and higher  $Z'$  values than the standard fluo-4 assay, while providing an easier and faster assay as well. The fluo-4 NW indicator is nonfluorescent and stable in pH 7–7.5 buffer for several hours, so spontaneous conversion to the  $\text{Ca}^{2+}$ -sensitive form is not a significant source of background fluorescence. Contributions to baseline fluorescence by the growth medium (e.g., esterase activity, proteins interacting with receptors of interest, or phenol

red) are eliminated by removing the medium prior to adding the indicator dye to the wells. Another source of potential fluorescence outside the cells is extrusion of the indicator out of the cell by organic anion transporters. Probenecid is commonly used to inhibit this transport and reduce the baseline signal. We have synthesized a proprietary water-soluble probenecid, which is supplied with the Fluo-4 NW Calcium Assay Kits. This form of probenecid has the advantages of being easy to dissolve in buffer and safer to use than the free acid, which requires caustic 1 M NaOH to dissolve. The Fluo-4 NW Calcium Assay Kits are designed for microplates and HTS, and the assay can be performed on adherent as well as non-adherent cells.

### Materials

- **Fluo-4 NW dye mix** (Component A)
- **Probenecid, water soluble** (Component B)
- **Assay buffer** (1X HBSS, 20 mM HEPES) (Component C, included in starter pack only)

#### Materials Needed but Not Included (for high-throughput pack only):

- **1X Hanks' balanced salt solution (HBSS)**, 1.5 L (3 units of GIBCO catalog #14025-092, 500 mL each)
- **1 M HEPES buffer solution**, 30 mL (2 units of GIBCO catalog #15630-106, 20 mL each)

The Fluo-4 NW Calcium Assay Kit starter pack with buffer (F36206) contains enough materials for 10 microplates and includes assay buffer (Component C). The Fluo-4 NW Calcium Assay Kit for high throughput (F36205) contains enough materials for 100 microplates and does not include the assay buffer.

### General Recommendations

Prepare reagents on the day that you will perform your experiments. Ensure that Components A and B are completely dissolved when making the reagent solutions. Reagent quantities are given for assaying one microplate at a time (starter pack) or ten microplates at a time (high-throughput kit); scale up quantities according to your experiment. **Experiments with partial plates (for the starter pack) or numbers of plates other than multiples of 10 (for the high-throughput kit) are not recommended, as the dye loading solution can only be used on the day it is prepared.**

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## Protocol for Adherent Cells

### A. Preparation of Cells

**1.1** Culture adherent cells in 96- or 384-well microplates (poly-D-lysine-coated plates recommended), to near confluence. In our tests we used the M1WT3 (CHO M1) cell line (ATCC #CRL-1985) and the GripTite™ 293 MSR (HEK293) cell line (Invitrogen catalog #R795-07). In 96-well plates, CHO, HeLa, or 3T3 cells can be plated at 30,000–40,000 cells per well and grown overnight. HEK293 cells can be plated at 40,000–50,000 cells per well and grown overnight. If you are using 384-well plates, plate ¼ to ½ of these cell numbers per well.

### B. Preparation of Reagents

The high-throughput kit does not include assay buffer (see step 1.2). If you are using the starter pack, the assay buffer is provided as Component C; skip to step 1.3. **Note:** The dye solution, made from Component A (see step 1.4), must be used on the same day it is made. To minimize waste of the dye solution, we recommend experiments with full plates (for the starter pack), or multiples of 10 plates (for the high-throughput kit).

**1.2** If you are using the high-throughput kit, make the quantity of assay buffer you will need, based on the number of microplates in your experiment (a minimum of 10 plates is assumed). For 10 microplates, a total of 150 mL of assay buffer will be sufficient for all reagent preparations. Make 150 mL of assay buffer by adding 3 mL of 1 M HEPES to 147 mL of 1X HBSS.

**1.3** Make a 250 mM stock solution of probenecid by adding 1 mL of assay buffer to one vial of probenecid (Component B). Vortex until dissolved. If this stock solution is not to be used the same day, it can be stored at ≤–20°C for up to 6 months.

**1.4** Make the dye loading solution for the kit that you are using, as follows:

**Starter pack:** Add **10 mL** of assay buffer and **100 µL** of the probenecid stock solution to one bottle of Component A. This 1X dye loading solution is sufficient for one microplate, and the probenecid concentration is 2.5 mM.

**High-throughput kit:** Add **100 mL** of assay buffer and **1 mL** of the probenecid stock solution to one bottle of Component A. This 1X dye loading solution is sufficient for ten microplates, and the probenecid concentration is 2.5 mM.

**Note:** Shake or vortex the dye loading solution vigorously for 1–2 minutes to ensure that the dye dissolves completely.

**1.5** Prepare a solution of the receptor agonist for your experiment in assay buffer (without probenecid).

### C. Assay

**1.6** Remove the growth medium from the adherent cell cultures. It is very important to remove the growth medium in order to eliminate sources of baseline fluorescence, particularly esterase activity. Quickly but carefully add 100 µL of the dye loading solution to each well of a 96-well plate, or 25 µL per well of a 384-well plate.

**1.7** If the calcium response will be measured (in step 1.8) at room temperature, incubate the plate(s) at 37°C for 30 minutes, then at room temperature for an additional 30 minutes. We have found that the best results are obtained with this combination of times and temperatures for incubation and measurement. Alternatively, if the response will be measured at 37°C, incubate the plate(s) for 30–45 minutes at 37°C (without further incubation at room temperature). The plates are now ready to be used in an experiment. **It is not necessary to remove the dye loading solution from the wells.**

**1.8** Measure fluorescence using instrument settings appropriate for excitation at 494 nm and emission at 516 nm. A 488 nm argon laser line works well.

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## Protocol for Non-adherent Cells

### A. Preparation of Cells

Prior to pelleting the cells (step 2.3), ensure that you have sufficient assay buffer for the day's experiments. The high-throughput kit does not include assay buffer (see step 2.1). If you are using the starter pack, the assay buffer is provided as Component C; skip to step 2.2.

**2.1** If you are using the high-throughput kit, make the quantity of assay buffer you will need, based on the number of microplates in your experiment (a minimum of 10 plates is assumed). For 10 microplates, a total of 150 mL of assay buffer will be sufficient for all reagent preparations. Make 150 mL of assay buffer by adding 3 mL of 1 M HEPES to 147 mL of 1X HBSS.

**2.2** Count the density of cells directly from the culture flask. Calculate the volume of culture that will yield 125,000 cells per well in a 96-well plate, or 31,250 cells per well in a 384-well plate. These suggested cell densities have been found to work well for a Jurkat cell line (GeneBLAzer® muscarinic receptor (M1) NFAT-*bla* Jurkat cell line, Invitrogen catalog #K1051).

**Example:** density of cell culture =  $1.5 \times 10^6$  cells/mL

For each microplate (~100 wells in 96-well plates or ~400 wells in 384-well plates) you will need:

$125,000 \text{ cells} \times 100$  (or  $31,250 \text{ cells} \times 400$ ) =  $1.25 \times 10^7$  cells

$\frac{1.25 \times 10^7 \text{ cells}}{1.5 \times 10^6 \text{ cells/mL}} = 8.3 \text{ mL of culture required}$

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

**2.4** Remove the medium from the cell pellet, and resuspend the pellet in assay buffer to a density of  $\sim 2.5 \times 10^6$  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 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 will fill.

### Example:

50  $\mu\text{L}$  per well  $\times$  100 wells in 96-well plates = 5 mL  
12.5  $\mu\text{L}$  per well  $\times$  400 wells in 384-well plates = 5 mL

**2.5** Pipet the resuspended cells, 50  $\mu\text{L}$  per well or 12.5  $\mu\text{L}$  per well, into the microplate(s). If desired, pipet the same volume of the assay buffer alone into no-cell control wells.

**2.6** Incubate the plate(s) at 37°C and 5% CO<sub>2</sub> for 60 minutes to allow the cells to settle.

### B. Preparation of Reagents

**Note:** The dye solution, made from Component A (see step 2.8), must be used on the same day it is made. To minimize waste of the dye solution, we recommend experiments with full plates (for the starter pack), or multiples of 10 plates (for the high-throughput kit).

**2.7** Make a 250 mM stock solution of probenecid by adding 1 mL of assay buffer to one vial of probenecid (Component B). Vortex until dissolved. If this stock solution is not to be used the same day, it can be stored at  $\leq -20^\circ\text{C}$  for up to 6 months.

**2.8** Make a 2X dye loading solution for the kit that you are using, as follows:

**Starter pack:** Add 5 mL of assay buffer and 100  $\mu\text{L}$  of the probenecid stock solution to one bottle of Component A. This 2X dye loading solution is sufficient for one microplate, and the probenecid concentration is 5 mM.

**High-throughput kit:** Add 50 mL of assay buffer and 1 mL of the probenecid stock solution to one bottle of Component A. This 2X dye loading solution is sufficient for ten microplates, and the probenecid concentration is 5 mM.

**Note:** Shake or vortex the dye loading solution vigorously for 1–2 minutes to ensure that the dye dissolves completely.

**2.9** Prepare a solution of the receptor agonist for your experiment in assay buffer (without probenecid).

### C. Assay

**2.10** Remove the plate(s) containing the cells from the incubator, and add 50  $\mu\text{L}$  of the 2X dye loading solution to each well of a 96-well plate, or 12.5  $\mu\text{L}$  per well of a 384-well plate.

**2.11** If the calcium response will be measured (in step 2.12) at room temperature, incubate the plate(s) at 37°C for 30 minutes, then at room temperature for an additional 30 minutes. We have found that the best results are obtained with this combination of times and temperatures for incubation and measurement. Alternatively, if the response will be measured at 37°C, incubate the plate(s) for 30–45 minutes at 37°C (without further incubation at room temperature). The plates are now ready to be used in an experiment. **It is not necessary to remove the dye loading solution from the wells.**

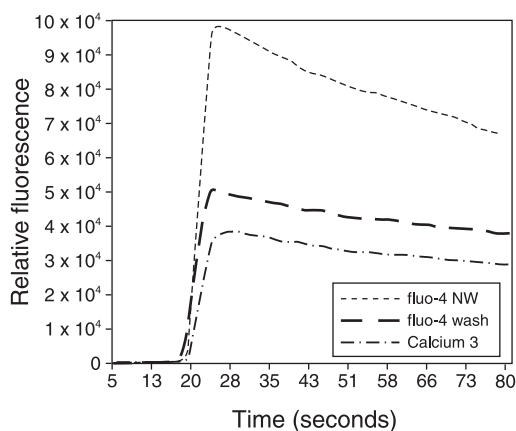
**2.12** Measure fluorescence using instrument settings appropriate for excitation at 494 nm and emission at 516 nm. A 488 nm argon laser line works well.

### Typical Results

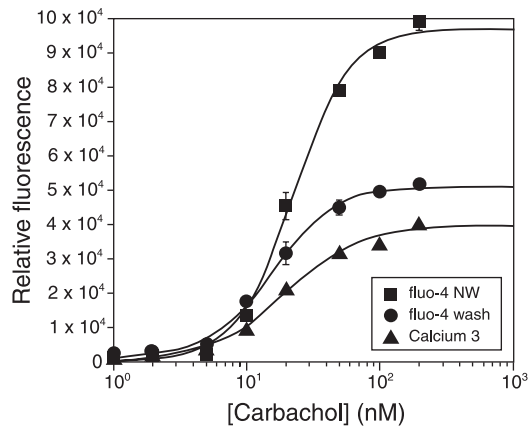
In a side-by-side comparison of the fluo-4 NW assay, the standard fluo-4 assay with wash, and the Calcium 3 (Molecular Devices) assay, CHO M1 cells were stimulated with a maximal concentration (200 nM) of carbachol. The fluo-4 NW assay showed a significantly greater fluorescence increase than the other assays (Figure 1). The larger response with the fluo-4 NW assay was accompanied by higher baseline fluorescence ( $\sim 26,000$  RFU vs.  $\sim 13,000$  RFU for the other two assays), which is likely due to the increased loading of cells with the dye in the fluo-4 NW assay. Baseline fluorescence can easily be subtracted digitally. In HEK293 cells and Jurkat cells, the improved performance was not as dramatic as in the CHO cells, but the fluo-4 NW assay did give slightly larger fluorescence increases, lower variability, and moderately better Z' scores compared to the Calcium 3 assay.

Dose responses for carbachol in CHO M1 cells gave similar EC<sub>50</sub> for all three assays, indicating consistent pharmacology (Figure 2). A four-parameter fit to each data set gave EC<sub>50</sub> = 22.6 nM for the fluo-4 NW assay, EC<sub>50</sub> = 15.9 nM for the standard fluo-4 assay, and EC<sub>50</sub> = 19.7 nM for the Calcium 3 assay. With HEK293 cells stimulated with carbachol, EC<sub>50</sub> values were 13.6  $\mu\text{M}$ , 14.1  $\mu\text{M}$ , and 19.0  $\mu\text{M}$  for the fluo-4 NW, standard fluo-4, and Calcium 3 assay, respectively. Using Jurkat cells and carbachol as an agonist, EC<sub>50</sub> values measured with the fluo-4 NW and Calcium 3 assays were 13 nM and 12 nM, respectively.

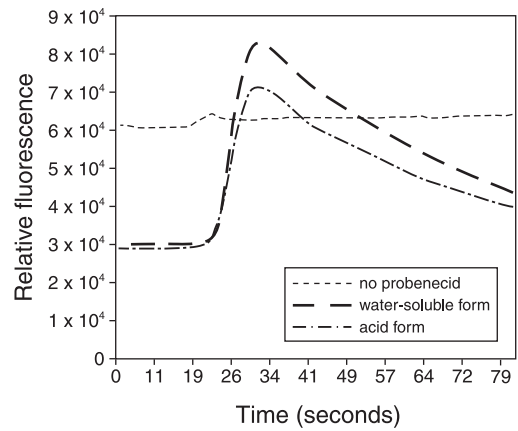
The water-soluble form of probenecid provided in the fluo-4 NW assay kits is easier and safer to use than the conventionally used free acid form, and it effectively inhibits extrusion of dye from the cells (Figure 3).



**Figure 1.** Comparison of measured fluorescence response using the fluo-4 NW, standard fluo-4, and Calcium 3 assays. CHO cells stably expressing the M1 muscarinic receptor were grown on poly-D-lysine-coated 96-well plates. The fluo-4 NW and Calcium 3 indicators were used according to manufacturers' instructions, and the standard fluo-4 AM indicator was used at 4  $\mu\text{M}$  with a wash step to remove the dye loading solution. For all three assays, cells were loaded for 30 min at 37°C followed by 30 min at room temperature. Cells were stimulated with 200 nM carbachol, and responses were measured in a FlexStation II<sup>384</sup> fluorescence reader (Molecular Devices). The averages of four baseline-subtracted measurements were plotted.



**Figure 2.** Dose response curves for carbachol in CHO M1 cells.



**Figure 3.** Inhibition of dye extrusion using 2.5 mM probenecid. CHO M1 cells were incubated in dye loading solution for 60 minutes at 37°C, which accentuates the probenecid effect. Calcium release was triggered in CHO M1 cells using 20 nM carbachol. The averages of four measurements (without baseline subtraction) in a fluo-4 NW assay were plotted.

## References

1. Cell Calcium 27, 97 (2000); 2. Biotechniques 34, 164 (2003).

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

Cat #	Product Name	Unit Size
F36205	Fluo-4 NW Calcium Assay Kit (high-throughput) *for 100 microplates*	1 kit
F36206	Fluo-4 NW Calcium Assay Kit (starter pack with buffer) *for 10 microplates*	1 kit
P36400	probenecid, water soluble	10 x 77 mg
F14202	fluo-4, AM *packaged for high-throughput screening*	5 x 1 mg

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