

*HD Biosciences (China) Co., Ltd*

Product Manual

**HDB Wash Free Fluo-8 Calcium Assay Kit**

HD03-0010

HD03-0100

## Introduction

Fluo-8 AM is currently the brightest calcium indicator, more than 2 fold brighter than Fluo-4 AM, and 4 times brighter than Fluo-3 AM. With Fluo-8, the signal intensity and assay robustness have been greatly improved. Dye loading can be performed at room temperature. The Fluo-8 Calcium Assay Kit from HDB provides a fast, simple and reliable fluorescence-based assay for detecting changes in intracellular calcium. With this kit, calcium assays on fluorometric plate reader become a mix-and-read procedure in which cells are incubated with the kit reagents for one hour and transferred directly to plate reader for evaluation. There are no intermediate wash-steps involved.

## Applications

The kit will provide a homogeneous assay for calcium flux. It is especially designed to work for a broad range of targets, including GPCRs and ion channels.

## Materials and Equipments

### Kit Components

The following table lists the kit components.

Table 1: Each WASH Free Fluo-8 Calcium Assay Kit contains the following components. Each kit is sufficient for 10 or 100 plates (96-well or 384-well).

Reagent	Description
Component A	One vial lyophilized Fluo-8 AM
Component B	One vial lyophilized Component B
Component C	One bottle HBSS buffer (1x Hanks' BSS with 20 mM HEPES, pH 7.4) 100ml

### Reagent Required but Not Provided:

Probenecid (Sigma, Cat # P8761 )

## Storage and Handling

Upon receipt of the kit, remove the HDB Component A and store at <-20°C. Component B can be stored at room temperature. Store Component C at 4°C. Under the condition described above, the reagents are guaranteed to be stable for six months in the original packaging.

## HDB Fluo-8 Calcium Assay Kit Experimental Protocol

### Cell Handling

The HDB Fluo-8 Calcium Assay Kit is designed to work with many cell types, both adherent and non-adherent. In this section we provide guidelines on how to set up the cells for use with the assay kit.

Optimal cell conditions for the HDB Fluo-8 Calcium Assay Kit require creation of a confluent cell monolayer before placing the plates in plate reader. In general, we recommend starting with 30,000 cells/well for a 96-well plate. (If 384-well plate is used with the Calcium Assay Kit, 10,000 cells/well is recommended).

For adherent cells, we recommend seeding cells overnight with a plating volume of 100  $\mu$ l/well for 96-well plates (or 25  $\mu$ l/well for 384 well plates).

For non-adherent cells, we recommend a centrifugation of the cells from culture medium and suspension of the pellet in culture medium. A volume of 100  $\mu$ l (96-well plate) or 25  $\mu$ l (384-well plate) of cell suspension is added into each well. It is recommended that a centrifugation of the plates at 1000 rpm for up to 4 minutes is performed (*Note: with brake off*).

### 1. Preparation of 1 X Fluo-8 Calcium Assay Loading Solution

The following procedure is designed for one 96 or one 384-well plate using either adherent or non-adherent cells prepared as described above.

- Thaw 1 vial of Component A, and equilibrate 1 bottle of Component C at room temperature before use.
  
- **2 mM Component A (Fluo-8 AM) stock solution preparation**
  - a). For **10 plates kit**, dissolve 1 vial of Component A in 210  $\mu$ l DMSO, mix well.
  - b). For **100 plates kit**, dissolve 1 vial Component A in 2100  $\mu$ l DMSO, mix well.Store at  $<-20$  °C as aliquots and added freshly to the Loading Buffer at a final in-well working concentration of 4  $\mu$ M.  
*Note: 20  $\mu$ l of reconstituted Fluo-8 is enough for 1 plate, un-used reconstituted Fluo-8 can be aliquoted and stored at  $<-20$ °C for six months if the tubes are sealed tightly, avoiding light and repeated freeze-thaw cycles.*
  
- **50 X Component B stock solution preparation**
  - a). For **10 plates kit**, dissolve 1 vial of Component B in 2.4 ml Component C, mixing them well.
  - b). For **100 plates kit**, dissolve 1 vial Component B in 24 ml Component C, mixing them well.*Note: 200  $\mu$ l of reconstituted Component B is enough for 1 plate. Un-used reconstituted Component B can be aliquoted and stored at room temperature for six months if the tubes are sealed tightly, avoiding light.*
  
- **200mM probenecid stock solution preparation**  
Dissolve probenecid in 200mM NaOH.

*Note:* All HBSS buffer used below containing 2mM probenecid, including Fluo-8 calcium assay loading solution and buffer to prepare agonist, etc. This solution is prepared fresh by diluting 200mM probenecid stock solution (Dissolved in 200mM NaOH) into HBSS Buffer. Store at -20 °C as aliquots and added freshly to the Loading Buffer at a final in-well working concentration of 2 mM.

➤ **1X Fluo-8 Calcium Assay Loading Solution preparation**

**a).** For **10 plates kit**, make 10 ml of 1X Fluo-8 Calcium Assay Loading Solution as described below:

- ♦ 20 µl 2 mM Fluo-8 AM
- ♦ 200 µl 50 X component B
- ♦ 100 µl 200 mM probenecid
- ♦ 9680 µl Component C

**b).** For **100 plates kit**, make 100 ml of 1X Fluo-8 Calcium Assay Loading Solution as described below:

- ♦ 200 µl 2 mM Fluo-8 AM
- ♦ 2 ml 50 X component B
- ♦ 1 ml 200 mM probenecid
- ♦ 96.8 ml Component C

*Note:* Mix them well. 10ml 1X Fluo-8 calcium assay loading solution is enough for one plate. Do not store frozen aliquots of Loading Solution with Fluo-8 or probenecid; always add fresh Fluo-8 and probenecid on the day of experiment.

**2. Loading cells using Loading Buffer**

- Remove cell plates from incubator or centrifuge. Remove the growth medium from cell plates. Add 100 µl 1X Fluo-8 Calcium Assay Loading Solution per well for 96-well plates, 25µl per well for 384 well plates.

*Note:* It is recommended to remove the growth medium in order to minimize background fluorescence, and compound interference with serum or culture media. Alternatively, one can grow the cells in growth medium with 0.5–to 1% FBS to avoid medium removal step, in this case, make 2X Fluo-8 Calcium Assay Loading Solution in HBSS buffer.

- Incubate cell plates for 30 minutes at 37 °C and then keep the plates at room temperature for another 30 minutes until use.

*Note:* In some cases, the assay requires 37°C. Perform the experiment immediately after the 1 hour 37°C incubation without further room temperature incubation. The incubation time should be limited in 2 hours.

### 3. Running Calcium Assay on Plate Reader

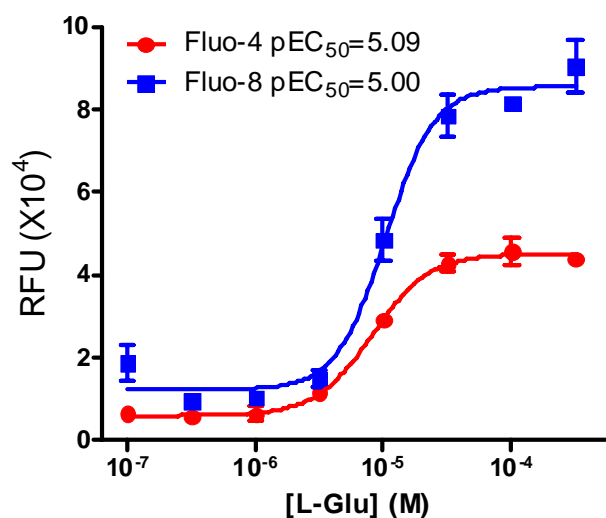
*Note: The HDB Fluo-8 Calcium Assay Kit is optimized for an agonist addition at one-fifth of the final volume. The agonist should be diluted with HBSS buffer (containing 2 mM freshly added probenecid, same as the concentration of probenecid in 1X Fluo-8 Calcium Assay Loading Solution).*

- Recommended experimental setup parameters:

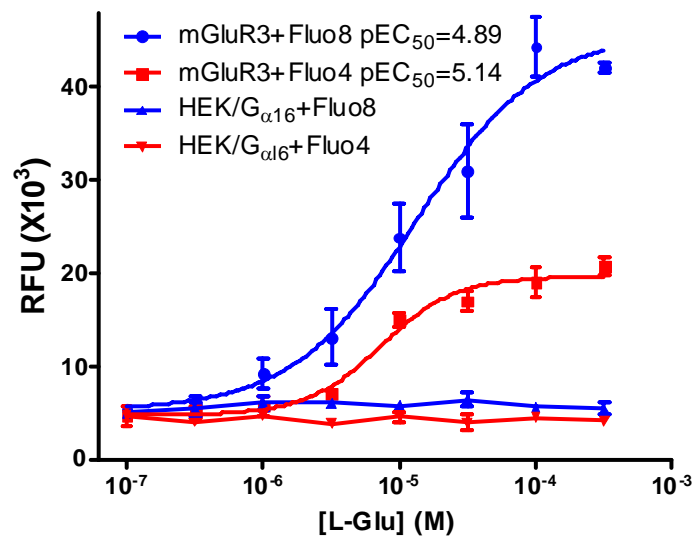
Parameters	
Excitation wavelength (nm)	490
Emission wavelength (nm)	525
Emission cut-off (nm)	515

- After incubation, transfer assay plates directly to a plate reader and run the assay. The assay should be completed within 3 to 5 min after compound addition. Analyze the data.

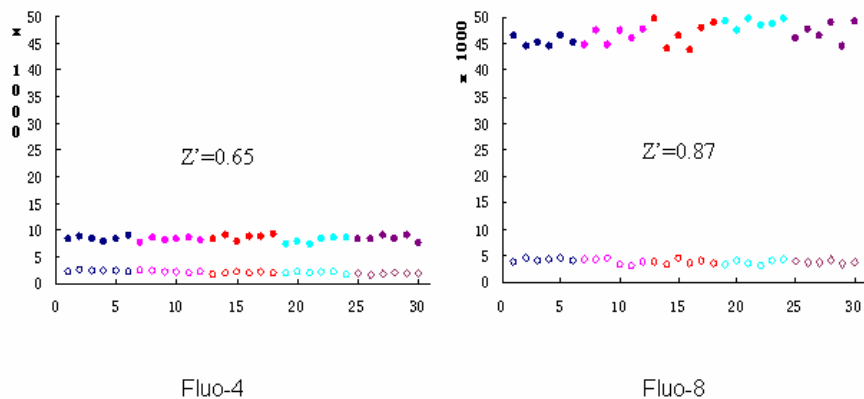
### Comparison Between Fluo-4 and Fluo-8 Calcium Assay Data



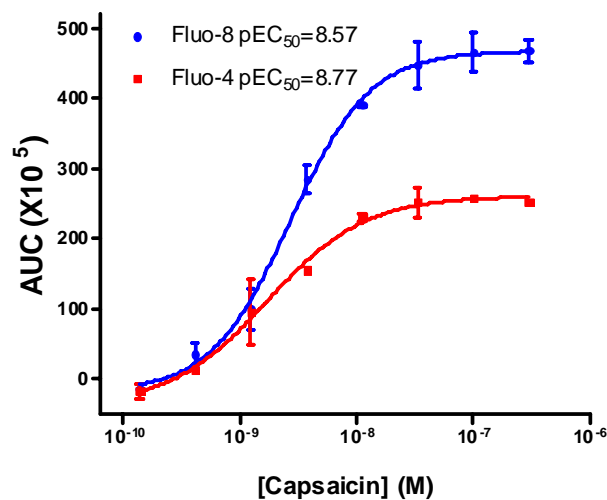
**Figure 1.** HEK293/mGluR6/Ga15 cells were seeded overnight in 100 $\mu$ l on a 96-well Matri-gel coated plate. Assays with Wash Free HDB Fluo-4 and Fluo-8 calcium assay kits were performed according to the protocols described in the kit manuals. Data points represent means  $\pm$  SEM. pEC<sub>50</sub> value was determined using GraphPad Prism 5 software.



**Figure 2.** HEK293/mGluR3/Ga16 and HEK293/Ga16 cell lines were seeded overnight in 100  $\mu$ l on a 96-well Matri-gel coated plate. Assays with Wash Free HDB Fluo-4 and Fluo-8 NW calcium assay kits were performed according to the protocols described in the kit manuals. Data points represent means  $\pm$  SEM.  $pEC_{50}$  value was determined using GraphPad Prism 5 software.



**Figure 3.** Z' factor determination in 96-well format for HEK293/mGluR3/Ga16 cells were performed with Wash Free HDB Fluo-4 and Fluo-8 NW calcium assay kits. HEK293/mGluR3/Ga16 cell line was stimulated with 100  $\mu$ M L-Glutamate (signal) and assay buffer (background).



**Figure 4.** HEK293/TRPV1 cells were seeded overnight in 100µl on a 96-well Matri-gel coated plate. Assays with Wash Free HDB Fluo-4 and Fluo-8 NW calcium assay kits were performed according to the protocols described in the kit manuals. Data points represent means  $\pm$  SEM. pEC<sub>50</sub> value was determined using GraphPad Prism 5 software.

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