Product Manual

GFP-CERTIFIED® FLUOFORTE® Calcium Assay Kit

Orange FLUOFORTE® Dye

Catalog Number: ENZ-51020-KP010
Starter Pack, for 10 x 96-well plates

Catalog Number: ENZ-51020-KP100
High-Throughput, for 100 x 96-well plates

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Product Manual

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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INTRODUCTION

The calcium ion is an important second messenger involved in many physiological and signal transduction processes within cells. Rhod-2 has been the most popular red fluorescent Ca^{2+} indicator for in-cell measurement of agonist-stimulated and antagonist-inhibited calcium signaling in high-throughput screening applications.[1-4] However, its relatively weak fluorescence signals have limited its application in some challenging cell lines and with certain membrane receptors.

Enzo Life Sciences' GFP CERTIFIED® FLUOFORTE® Calcium Assay Kit provides a homogeneous fluorescence-based assay for detecting intracellular calcium mobilization across a broad spectrum of biological targets. Relative to other commercially available red fluorescent dyes, GFP CERTIFIED® FLUOFORTE® dye is the brightest and most sensitive fluorescent calcium indictor. The kit provides a homogeneous mix-and-read, no-wash calcium mobilization assay. The homogenous cell-based assay for calcium offers fewer steps, lower variability and an easier protocol for adherent and non-adherent cell lines. In addition, it requires neither a washing step, nor exogenous addition of a quencher dye, which could adversely effect receptor-ligand interaction kinetics.[5]

SAFETY WARNINGS & PRECAUTIONS

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Handle with care

- This product is for research use only and is not intended for diagnostic purposes.
- Some components of this kit may contain hazardous substances. They can be harmful if ingested or absorbed through the skin and may cause irritation to the eyes. The reagents of the kit should be treated as possible mutagens and should be handled with care and disposed of properly.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. All blood components and biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.
- To avoid photobleaching, perform all manipulations in low light environments or protected from light by other means.

REAGENTS PROVIDED AND STORAGE

All reagents are shipped on dry ice. Upon receipt, the kit should be stored at -20°C, protected from light. When stored properly, these reagents are stable for six months from date of receipt. **Avoid repeated freezing and thawing**.

Reagent	Quantity	
	ENZ-51020-KP010 (for 10 plates)	**ENZ-51020-KP100 (for 100 plates)**
Reagent A: GFP CERTIFIED® FLUOFORTE® dye, lyophilized	1 vial	10 vials
Reagent B: Dye efflux inhibitor	10 x 1 mL	10 x 10 mL
Reagent C: Hanks' buffer with 20 mM HEPES (HHBS)	1 x 100 mL	Not included

ADDITIONAL MATERIALS REQUIRED

1. A fluorometric imaging plate reader capable of performing quantitative optical screening for cell-based kinetic assays. (Molecular Devices FLIPR, PerkinElmer CellLux, Hamamatsu FDSS system, or similar instrumentation)
2. Calibrated, adjustable precision pipetters, preferably with disposable plastic tips
3. Deionized water
4. Anhydrous DMSO
5. Serum (optional).
6. Growth medium (e.g. Dulbecco's modified Eagle medium, D-MEM)
7. Assay Plates: 96- or 384-well black-wall, clear bottom plates or 1536-well low base black-wall, clear bottom plates, 1536-well lids
8. Compound plates: 96-well or 384-well polypropylene plates, 1536-well polystyrene plates

METHODS AND PROCEDURES

Brief Summary of Assay Work Flow

- Prepare cells.
- Remove medium.
- Add GFP CERTIFIED® FLUOFORTE® dye loading solution.
- Incubate plate for 1 hour.
- Add test agents.
- Monitor fluorescence at Ex/Em=530/570 nm

***NOTE**: **PLEASE READ THE ENTIRE PROCEDURE BEFORE STARTING**. Allow all reagents to be used to warm to room temperature before proceeding. Upon thawing of solutions, gently hand-mix or vortex the reagents prior to use to ensure a homogenous solution.*

CELL PREPARATION

1. **Adherent Cells**. The day before the experiment, plate the cells overnight in growth medium using 4 x 10^4 to 8 x 10^4 cells per well at a plating volume of 100 µL per well for 96-well plates, or using 1 x 10^4 to 2 x 10^4 cells per well at a plating volume of 25 µL per well for a 384-well plates.

   After overnight incubation, remove the growth medium from the cell plates. Then proceed to Calcium Mobilization Assay section, page 6.

   ***NOTE**: It is important to remove the growth medium in order to minimize background fluorescence, and compound interference with serum or culture media.*

2. **Non-adherent Cells**. On the day of the experiment, centrifuge the cells from the culture medium and then resuspend the cell pellets in GFP CERTIFIED® FLUOFORTE® dye-loading solution (see page 5). Plate the cells using 1.25 x 10^5 to 2.5 x 10^5 cells per well at a plating volume of 100 µL per well for 96-well plates, or 3 x 10^4 to 6 x 10^4 cells per well at a plating volume of 25 µL per well for 384-well plates. Centrifuge the plates at 800 rpm for 2 minutes, **with brake off**, prior to starting the experiments. Proceed to Calcium Mobilization Assay section, page 6.

   ***NOTE**: Each cell line should be evaluated on an individual basis to determine the optimal cell density for the intracellular calcium mobilization assay.*

PREPARATION OF GFP CERTIFIED® FLUOFORTE® DYE LOADING SOLUTION

The following procedure is for preparation of GFP CERTIFIED® FLUOFORTE® dye loading solution for use in 1 plate. Before starting, equilibrate Reagent A (GFP CERTIFIED® FLUOFORTE® dye), a vial of Reagent B and Reagent C (HBSS) to room temperature.

1. **Reagent A Stock Solution**. Add 100 µL DMSO to the vial containing **Reagent A**. Mix well.

   ***NOTE**: 10 µL of the reconstituted Reagent A is enough for 1 plate. The remaining unused, reconstituted Reagent A can be aliquoted and stored at ≤ -20ºC for at least one month if stored properly. The tubes (preferably amber vials) should be capped tightly. Avoid exposure to light and repeated freeze-thaw cycles.*

2. **1X Assay Buffer**. Mix well 9ml of **Reagent C** (HHBS) with the contents of 1 vial of **Reagent B**.

   ***NOTE**: The **100 plates kit** does not include the HBSS Buffer. Be sure to prepare this reagent prior to starting any procedure.*

   10 mL of 1X Assay Buffer is sufficient for one plate. Unused buffer may be stored at ≤ -20°C up to 1 month. Avoid exposure to light and repeated freeze-thaw cycles.

3. **GFP CERTIFIED® FLUOFORTE® Dye Loading Solution**. Add 10 µL of Reagent A Stock Solution (from step 1 above) to 10 mL of 1X Assay Buffer (from 2 above). Mix well. This working solution is stable for at least **2 hours** at room temperature.

CALCIUM MOBILIZATION ASSAY

1. Obtain prepared cell plates (see Cell Preparation, page 4).
2. Add GFP CERTIFIED® FLUOFORTE® Dye Loading Solution to each well (100 µL/well (for 96-well plates) or 25 µL/well for 384-well plates).
3. Incubate the cell plates for 45 minutes in a 37°C cell incubator, and then incubate for another 15 minutes at room temperature.

   ***NOTE**: The incubation time should be optimized for each cell line. The incubation time should be limited to 1-2 hours. **DO NOT** wash the cells after dye loading.*
4. Prepare the compound plates by dissolving the compound in the buffer of choice. The GFP CERTIFIED® FLUOFORTE® Calcium Assay is optimized for an agonist addition at one-fifth of the final volume.
5. Run the calcium flux assay by monitoring the fluorescence at Ex=530 nm/Em=570 nm with a fluorometric imaging plate reader.

   ***NOTE**: Faster addition speeds can lead to better mixing of compounds and lower signal variance across the plate. Make sure to follow the recommended experimental setup parameters provided by the instrument manufacturer before reading the plate. It is also important to run the signal test before the experiment. Different instruments have their own intensity range. Adjust the signal test intensity to the level of 10% to 15% of the maximum instrument intensity counts. For example, the maximum fluorescence intensity count for FLIPR-384 is 65,000, so the instrument settings should be adjusted to have its signal test intensity around 7,000 to 10,000.*

CALCIUM MOBILIZATION MICROSCOPY ASSAY

1. Obtain prepared cell slides (see section A).
2. Add FLUOFORTE® Dye-Loading Solution to each region on the slide.
3. Incubate the cell slides for 1 hour at room temperature, or incubate about 45 minutes at 37°C then incubate for 15 minutes at room temperature prior to assay.

***NOTE:** The incubation time should be optimized for each cell line. The incubation time should be limited to 1~2 hours. **DO NOT** wash the cells after dye loading.*

4. Prepare the compound plates by dissolving the compound in the buffer of choice. The FLUOFORTE® Calcium Assay is optimized for an agonist addition between 1/5-1/6 of the total final volume (e.g., Add 20-25 µL/well of agent when using a 96-well plate that contains 100 µL of FLUOFORTE® Dye-Loading Solution[2]).
5. Run the calcium flux assay by monitoring the fluorescence with a fluorescence microscope with the appropriate filter.

CALCIUM MEASUREMENT IN FLOW CYTOMETRY

1. Aliquot between 1.25 x 10^5 to 2.5 x 10^5 cells per well at a volume of 250 µL in each polypropylene tube.

2. Add 250 µL FLUOFORTE® Dye-Loading Solution to each well.

3. Incubate the cell plates for 1 hour at room temperature, or incubate about 45 minutes at 37°C then incubate for 15 minutes at room temperature prior to assay.

NOTE: The incubation time should be optimized for each cell line. The incubation time should be limited to 1~2 hours. DO NOT wash the cells after dye loading.

4. Add agonist at desired concentration in small volume. Use a vehicle control.

5. Incubate the cell plates for 1 hour at room temperature, or incubate about 45 minutes at 37°C then incubate for 15 minutes at minutes at room temperature prior to assay.

NOTE: The incubation time should be optimized for each cell line. The incubation time should be limited to 1~2 hours. DO NOT wash the cells after dye loading.

6. Run samples on flow cytometer.

NOTE: Calcium flux is a transient event. The timing of the sample analysis should be consistent and may need optimization.

EXPECTED RESULTS

In a side-by-side comparison of GFP CERTIFIED® FLUOFORTE® and Rhod-2 AM dyes, CHO M1 cells were stimulated with 100nM of ATP. The GFP CERTIFIED® FLUOFORTE® dye yields much brighter signal. (shown in **Figure 1**). This enables calcium assays that are impossible with Rhod-2 AM and facilitates measurements of challenging cell lines and receptors.

Dose response curves for ATP in CHO M1 cells gave similar EC50 values (shown in **Figure 2**). This demonstrates consistent pharmacology among the assays. However, relative fluorescence units (delta RFU) of GFP CERTIFIED® FLUOFORTE® is much higher than Rhod2-AM and it has an approximately 10 times larger assay window than Rhod-2 AM for HTS applications.

Overall GFP CERTIFIED® FLUOFORTE® Calcium Assay kit provides an optimized assay method for monitoring G protein-coupled receptors (GPCRs) and calcium channels.[6] Its ability to generate very strong signal enables researchers to perform calcium mobilization assays with a wide range of receptor and calcium channel targets.

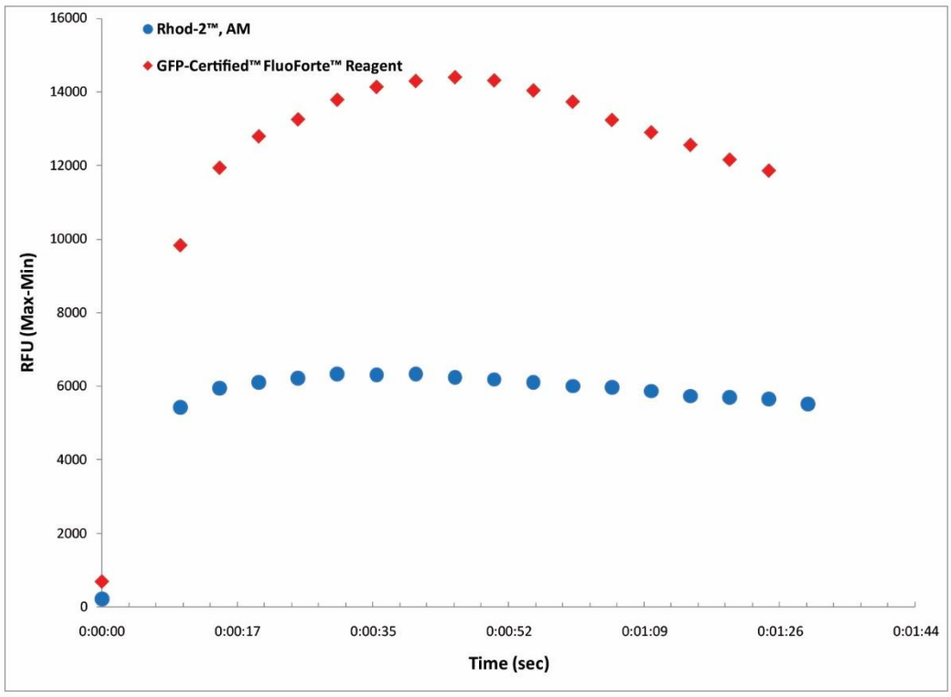

Figure 1: Comparisons of GFP CERTIFIED® FLUOFORTE® kit and Rhod-2 AM detection of intracellular calcium mobilization in CHO-M1 cells. CHO cells were seeded overnight in 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom Costar plate. The growth medium was removed, and cells were incubated with 100 µL of GFP CERTIFIED® FLUOFORTE® assay reagent, or 5 µM Rhod-2 AM for 1 hour at 37°C. ATP (20 µL/well) was added using a Biotek two syringe pump dispenser to achieve concentrations of 100 nM.

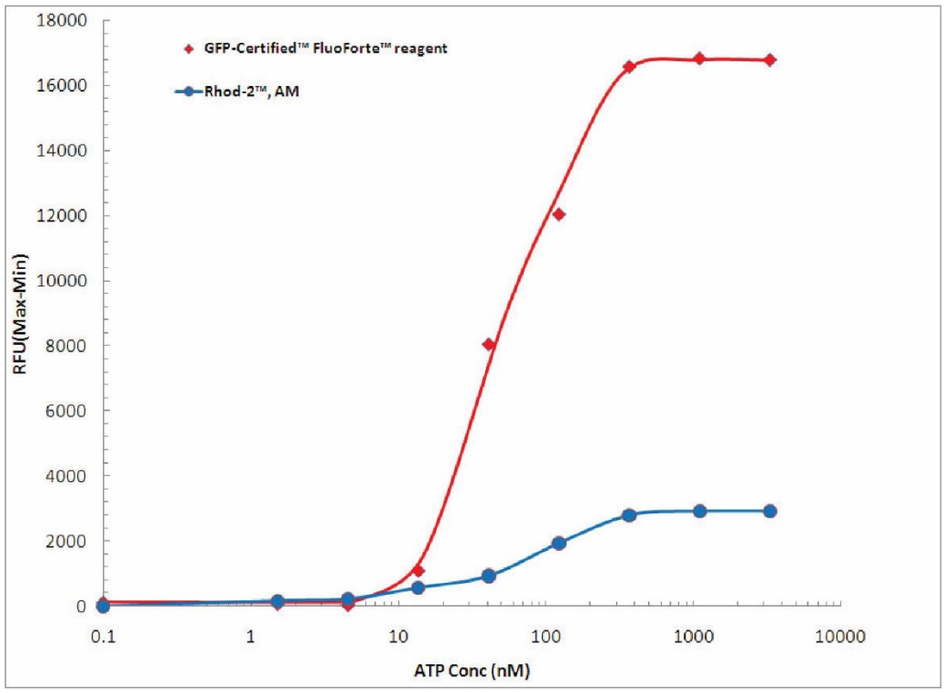

Figure 2: ATP Dose Response Curves in CHO-M1 cells. CHO cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom microplate. The cells were incubated with 100 µL of Enzo's GFP CERTIFIED® FLUOFORTE® dye and 5 µM Rhod-2 AM for 1 hour at 37°C. ATP (20 µL/well) was added a Biotek two syringe pump dispenser to achieve the final indicated concentrations. No significant difference in EC50 of ATP between GFP CERTIFIED® FLUOFORTE® and Rhod-2 AM was observed. The GFP CERTIFIED® FLUOFORTE® dye generated much higher intensity signal and larger assay window.

REFERENCES

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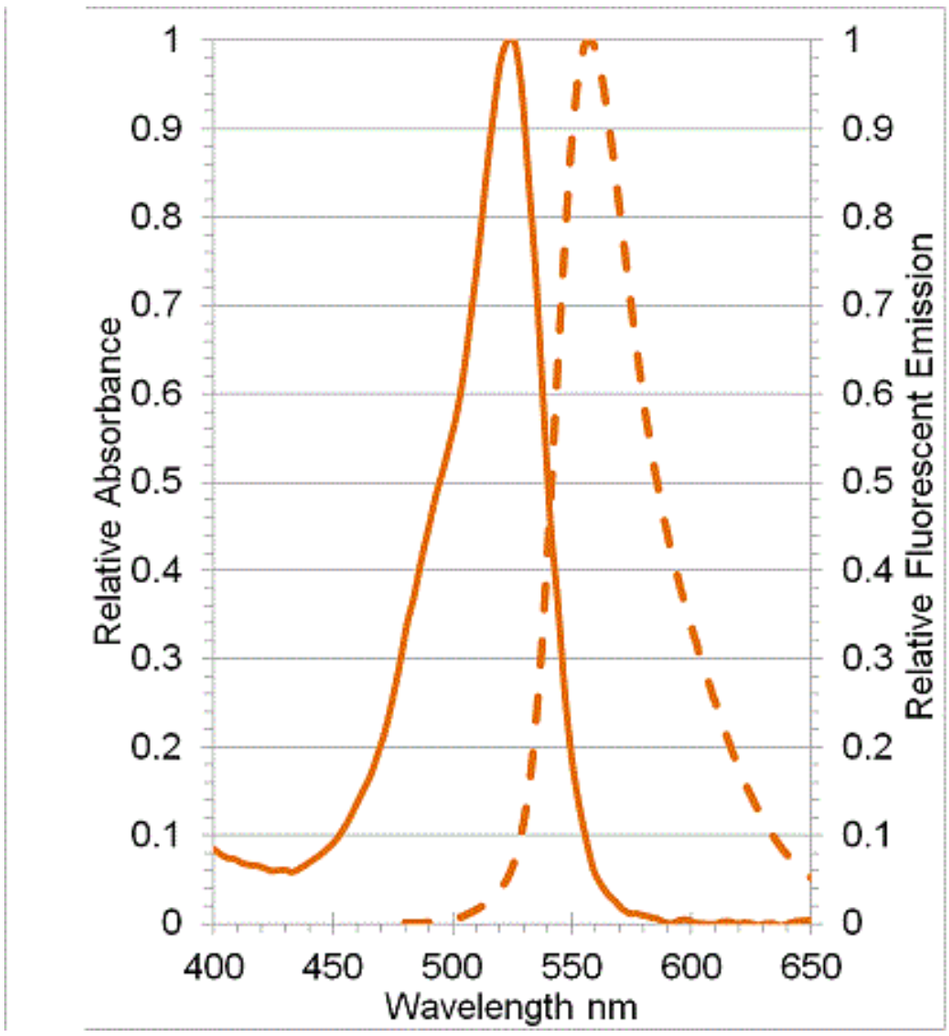
TROUBLESHOOTING GUIDE

Problem	Potential Cause	Suggestion
High baseline fluorescence	Contributions to baseline fluorescence by growth medium and organic anion transport	Remove the medium before adding the indicator dye to the wells. Use the dye solution within 2 hours at room temperature.
Untreated cells have calcium response	Inconsistent DMSO concentration	Make sure that the buffer used for the negative control wells have the same final concentration of DMSO as those in the test compounds.
Response is smaller than expected	Agonists and antagonists may stick to the pipette tips. Experimental setup parameters (Ex/Em wavelength) and dye loading time are not optimized.	Ensure Ex/Em =530/570 nm. Use 0.1% of BSA in all compound buffer diluents. Fast addition speeds are recommended to ensure better mixing of compounds and improved cell response. Dye loading typically takes between 30 minutes and one hour. Optimizing the conditions for each cell line is recommended.
Well to well variation observed	Incorrect dispenser and experimental setup parameter used.	Use instrument manufacturer's recommended dispenser and setup parameters (*i.e.,* volume, height and speed of dispensing) for compound addition.
Fluorescence drop upon compound addition	Dislodging the cells during addition	Decrease the rate of addition or seed fewer cells in the wells to avoid this problem.



Excitation and Emission Spectra for the GFP-CERTIFIED® FLUOFORTE® dye.

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