ab112128

Fluo-8 Medium Removal Calcium Assay Kit

Instructions for Use

For detecting calcium in cells by using our proprietary fluorescence probe

This product is for research use only and is not intended for diagnostic use.

Version 5b Last Updated 2 January 2024

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1. Introduction

Abcam Fluo-8 Medium Removal Calcium Assay Kit (ab112128) is a fluorescence-based assay for detecting intracellular calcium mobilization. Cells expressing a GPCR of interest that signals through calcium are pre-loaded with Fluo-8 which can cross cell membranes. Once inside the cell, the lipophilic blocking groups of Fluo-8 are cleaved by esterases, resulting in a negatively charged fluorescent dye that stays inside cells. Its fluorescence is greatly enhanced upon binding to calcium. When cells stimulated with agonists, the receptor signals the release of intracellular calcium, which significantly increase the fluorescence of Fluo-8. The characteristics of its long wavelength, high sensitivity, and >100 times fluorescence enhancement make Fluo-8 the brightest green calcium indicator available in the marker, and it is ideal tool for the measurement of cellular calcium through HTS screening.

ab112128 provide an optimized assay method for monitoring the G-protein-coupled receptors and calcium channels. The assay can be performed in a convenient 96-well or 384-well microtiter-plate format.

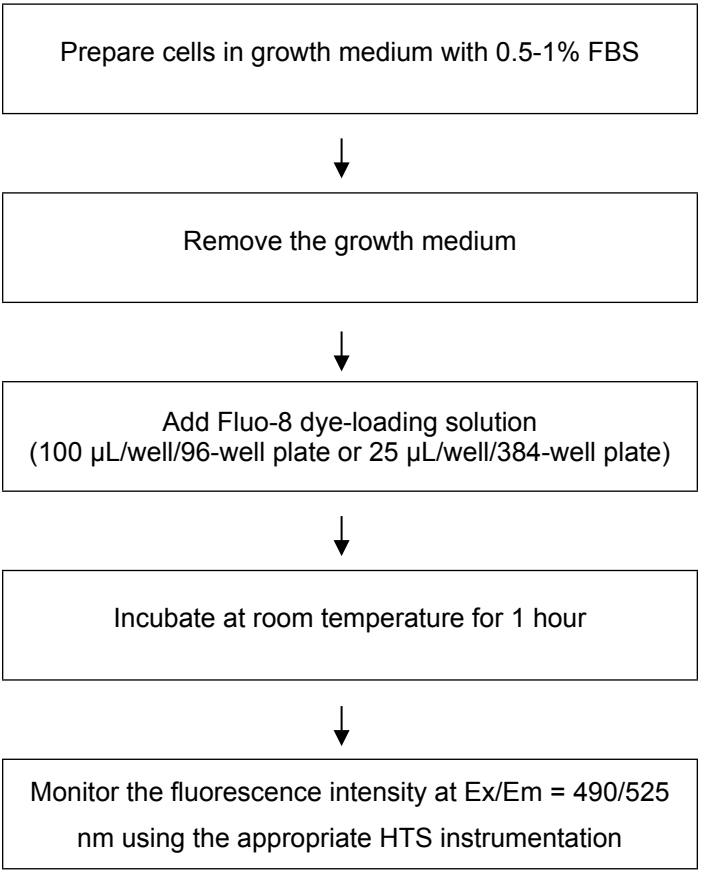
This product has been optimized for HTS screening. We recommend to use this product with the following imaging plate readers: FLIPR™, FDSS, BMG NOVOstar™, FlexStation, ViewLux, IN Cell Analyzer or Arrayscan.

Kit Key Features

- **B Increased Signal Intensity**: Fluo-8 is a green bright calcium indicator.
- **Flexible Dye Loading**: Dye loading at RT.
- **Convenient**: Formulated to have minimal hands-on time. No wash step needed.
- **Versatile Applications**: Compatible with many cell lines and targets without ligand or target interference.

2. Protocol Summary

Summary for One 96-well Plate; Warning: Do not add additional probenecid.



Note: Thaw all the kit components to room temperature before starting the experiment.

3. Kit Contents

Item	1 x 96 tests	10 x 96 tests	100 x 96 tests
Flou-8	1 vial (lyophilized)	1 vial (lyophilized)	10 vials (lyophilized)
10X Pluronic® F127 Plus	1 x 1 mL	10 x 1mL	10 x 10 mL
HHBS	1 x 9 mL	1 x 100 mL	Not Included

4. Storage and Handling

Keep at -20°C. Protect from light.

5. Additional Materials Required

- A 96 or 384-well microplate: Tissue culture microplate with black wall and clear bottom.
- A HTS fluorescence microplate reader with a filter set of Ex/Em = 490/525 nm. We recommend the following instruments: FLIPR™, FDSS, BMG NOVOstar™, FlexStation, ViewLux, IN Cell Analyzer or Arrayscan.
- HHBS (1X Hank's with 20 mM Hepes Buffer, pH 7.0).
- 100% DMSO
- (Optional) Carbachol (ab141354) or other GPCR agonists to induce agonist-mediated calcium release in cells.

6. Assay Protocol

Note: This protocol is for one 96 - well plate.

Warning: Do not add additional probenecid. It is recommended to incubate the dye loading solution no longer than 2 hours.

A. Prepare Cells

1. For adherent cells: Plate cells overnight in growth medium with 0.5-1% FBS at 40,000 to 80,000 cells/well/100 µL for a

96-well plate or 10,000 to 20,000 cells/well/25 µL for a 384-well plate.

2. For non-adherent cells: Centrifuge the cells from the culture medium and then suspend the cell pellet in equal amount of HHBS and Fluo-8 dye-loading solution (see Step B.4 below) at 125,000 to 250,000 cells/well/100 µL for a 96-well poly-D lysine plate or 30,000 to 60,000 cells/well/25 µL for a 384-well poly-D lysine plate. Centrifuge the plate at 800 rpm for 2 minutes with brake off prior to the experiments.

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

B. Preparation of Fluo-8 dye-loading solution

1. Thaw all the kit components at room temperature before use.

2. Make Fluo-8 stock solution: Add 10 µL or 100 µL of DMSO into Fluo-8 and mix them well.

   Note: 10 µL of Fluo-8 stock solution is enough for one plate. Un-used Fluo-8 stock solution can be aliquoted and stored at < -20°C for more than one month if the tubes are sealed

tightly. Keep from light and avoid repeated freeze-thaw cycles.

3. Make 1X assay buffer: For 1 x 96 tests and 10 x 96 tests kits make 1X assay buffer by adding 9 mL of HHBS into 1 mL of 10X Pluronic® F127 Plus, and mix them well.

   For 100 x 96 tests kits, make 1X assay buffer by adding the whole bottle of 10X Pluronic® F127 Plus (10 mL) into 90 mL of HHBS buffer (not included in the kit), and mix them well.

   Note: 10 mL of 1X assay buffer is enough for one plate. Aliquot and store un-used 1X assay buffer at < -20°C. Keep from light and avoid repeated freeze-thaw cycles.

4. Make Fluo-8 dye-loading solution for one cell plate: Add 10 µL of Fluo-8 stock solution (from Step 2) into 10 mL of 1X assay buffer (from Step 3), and mix them well. This working solution is stable for at least 2 hours at room temperature.

C. Run Calcium Assay:

1. Remove the growth medium from the cell plate.

Note 1: It is important to remove the growth medium in order to minimize background fluorescence, and compound interference with serum or culture media.

Note 2: Alternatively, grow the cells in growth medium with 0.5-to 1% FBS to avoid medium removal step. In this case, 2X dye loading solution in HHBS buffer is needed

2. Add 100 µL/well (96-well plate) or 25 µL/well (384-well plate) of Fluo-8 dye-loading solution (from Step B.4) into the cell plate (from step C.1)

3. Incubate the dye-loading plate in a cell incubator for 30 minutes, and then incubate the plate at room temperature for another 30 minutes.

Note 1: If the assay requires 37°C, perform the experiment immediately without further room temperature incubation.

Note 2: If the cells can function well at room temperature for longer time, incubate the cell plate at room temperature for 1-2 hours. (It is recommended that the incubation time be no longer than 2 hours.)

4. Prepare the compound plates with HHBS or the desired buffer.

5. Using your instrument that contains a pipettor (such as Flexstation from Molecular Devices), dispense compounds directly onto to the cell plate while collecting the data simultaneously (see next step for Ex/Em instructions).

Note: Typically the instrument collects the data every second for 100 seconds. Use the max signal to generate the plot. You can use a fluorescence microscope by adding the stimuli while taking the picture simultaneously.

6. Run the calcium flux assay by monitoring the fluorescence intensity at Ex/Em = 490/525 nm.

Note: It is important to run the signal test before your experiment. Different instruments have their own intensity

range. Adjust the signal test intensity to the level of 10% to 15% of the maximum intensity counts.

7. Data Analysis

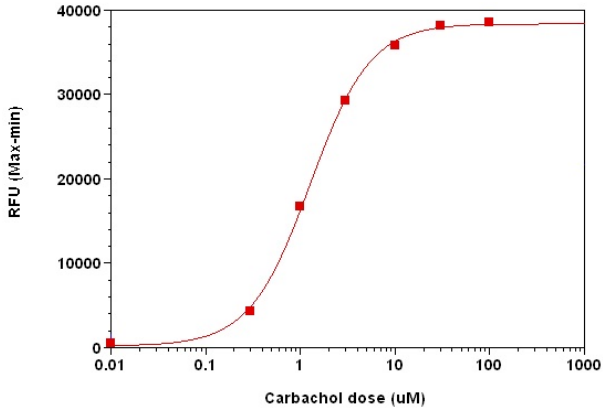


Figure 1. Carbachol Dose Response was measured in HEK-293 cells with ab112128. HEK-293 cells were seeded overnight at 40,000 cells/100 µL/well in a black wall/clear bottom 96-well plate. The growth medium was removed, and the cells were incubated with 100 µL of dye-loading solution using ab112128 (according to the manufacturer's instructions) for 1 hour at room temperature. Carbachol (25µL/well) was added to achieve the final indicated concentrations. The EC_{50} of Fluo-8 is about 1.2 µM.

8. Troubleshooting

Problem	Reason	Solution
Assay not working	Assay buffer at wrong temperature	Assay buffer must not be chilled - needs to be at RT
	Protocol step missed	Re-read and follow the protocol exactly
	Plate read at incorrect wavelength	Ensure you are using appropriate reader and filter settings (refer to datasheet)
	Unsuitable microtiter plate for assay	Fluorescence: Black plates (clear bottoms); Luminescence: White plates; Colorimetry: Clear plates. If critical, datasheet will indicate whether to use flat- or U-shaped wells
Unexpected results	Measured at wrong wavelength	Use appropriate reader and filter settings described in datasheet
	Samples contain impeding substances	Troubleshoot and also consider deproteinizing samples
	Unsuitable sample type	Use recommended samples types as listed on the datasheet
	Sample readings are outside linear range	Concentrate/ dilute samples to be in linear range

Problem	Reason	Solution
Samples with inconsistent readings	Unsuitable sample type	Refer to datasheet for details about incompatible samples
	Samples prepared in the wrong buffer	Use the assay buffer provided (or refer to datasheet for instructions)
	Samples not deproteinized (if indicated on datasheet)	Use the ***10kDa spin column (ab93349)*** or appropriate deproteinization protocol
	Cell/ tissue samples not sufficiently homogenized	Increase sonication time/ number of strokes with the Dounce homogenizer
	Too many freeze-thaw cycles	Aliquot samples to reduce the number of freeze-thaw cycles
	Samples contain impeding substances	Troubleshoot and also consider deproteinizing samples
	Samples are too old or incorrectly stored	Use freshly made samples and store at recommended temperature until use
Lower/ Higher readings in samples and standards	Not fully thawed kit components	Wait for components to thaw completely and gently mix prior use
	Out-of-date kit or incorrectly stored reagents	Always check expiry date and store kit components as recommended on the datasheet
	Reagents sitting for extended periods on ice	Try to prepare a fresh reaction mix prior to each use
	Incorrect incubation time/ temperature	Refer to datasheet for recommended incubation time and/ or temperature
	Incorrect amounts used	Check pipette is calibrated correctly (always use smallest volume pipette that can pipette entire volume)

Standard curve is not linear	Not fully thawed kit components	Wait for components to thaw completely and gently mix prior use
	Pipetting errors when setting up the standard curve	Try not to pipette too small volumes
	Incorrect pipetting when preparing the reaction mix	Always prepare a master mix
	Air bubbles in wells	Air bubbles will interfere with readings; try to avoid producing air bubbles and always remove bubbles prior to reading plates
	Concentration of standard stock incorrect	Recheck datasheet for recommended concentrations of standard stocks
	Errors in standard curve calculations	Refer to datasheet and re-check the calculations
	Use of other reagents than those provided with the kit	Use fresh components from the same kit

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