Molecular Devices

Product Insert

FLIPR® Calcium 4 Assay Kit

Product # R8142 (Explorer Kit)
R8141 (Bulk Kit)
R8143 (Express Kit)

About the FLIPR Calcium 4 Assay Kit

The Calcium 4 Assay Kit from Molecular Devices Corporation provides a fast, simple and reliable fluorescence-based assay for detecting changes in intracellular calcium. With this kit, as with all other kits from Molecular Devices based on its proprietary masking dye technology, calcium assays on FLIPR or FlexStation® become a mix-and-read procedure in which cells are incubated with the kit reagents for one hour and transferred directly to FLIPR or FlexStation for evaluation. There are no intermediate wash-steps involved.

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Introduction

About the FLIPR Calcium 4 Assay Kit

The Calcium assay kits from Molecular Devices employ sensitive Calcium indicators in AM form together with masking dyes. This mix is incubated 1h on the cells and then cells are ready to be assayed. The AM form of the calcium indicator enters the cells, the AM groups are cleaved and the dye is thus not able to diffuse out of the cell again. The masking dye does not enter the cell, but masks all residual extra cellular fluorescence of Calcium indicator, media and compounds, thus lowering the background significantly and enabling direct measurement of intracellular fluorescence change due to increased calcium concentration upon target activation.

Assay Principle

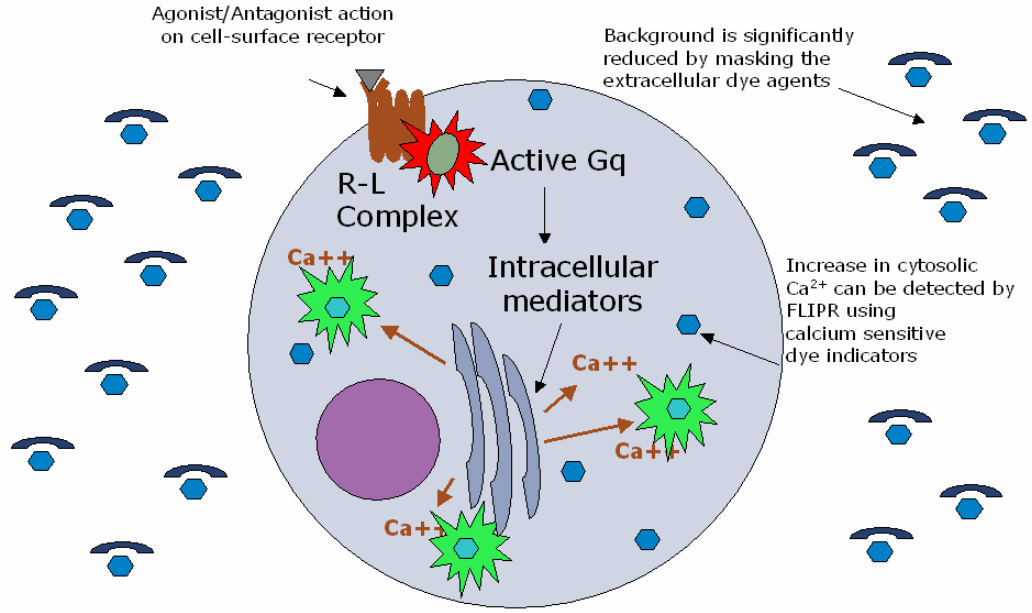

Figure 1: Calcium assay principle

Advantages

Conventional calcium mobilization protocols are multi-step procedures which begin by pre-washing the cells, loading them with a calcium indicator (e.g.: Fluo-3 or Fluo-4), followed by extensive cell washing prior to running the assay. This type of wash protocol can induce the following problems.

- Cells removed from plates during the wash procedure
- Reduced responsiveness (competence) of cells after washing due to perturbation
- Spontaneous calcium flux in the negative control cells upon buffer addition
- Variation in residual volume of wash buffer, leading to variation in the concentration of test compound
- Incomplete washing, resulting in a significant signal drop upon addition of test compound

- Molecular Devices developed its line of FLIPR Calcium Assay Kits to eliminate the cause of data variability and reduce the number of steps in the conventional wash protocol using Fluo-3 or Fluo-4. This homogenous approach introduces the following improvements over the wash protocol. (next page)

- Enhanced signal dynamic range
- Improved data quality
- Reduced well-to-well variation
- Ease of use with both adherent and non-adherent cells
- Rapid procedure with less hands-on time
- Fewer assay steps, resulting in higher sample throughput
- Minimal cell perturbation, reducing spontaneous calcium fluxes
- Broad range of applications for GPCR targets and calcium channels
- Adaptable for use in 96-, 384-, or 1536-well formats

The Calcium 4 kit uses a completely re-developed masking dye, which results in the following improvements for most cell systems over earlier Molecular Devices Calcium Assay kits.

- Increased assay signal,
- Reduced background,
- Increased z-factor
- Drastically reduced addition artifact (“dip”)

Applications

The kit provides a homogeneous assay for calcium flux. It is designed to work for the majority of GPCRs, including chemokine and other difficult receptors, as well as calcium channels.

Materials

Kit Components

Table 1: FLIPR Calcium 4 Assay Kit (P/N R8142, R8141, R8143) contents.

Reagent	Description
R8142(Explorer Kit)	• 10 vials Component A • 1 bottle Component B 1X Hank’s Balanced Salt solution (HBSS) plus 20 mM HEPES buffer, pH 7.4 • The entire kit is sufficient for ten 96-, 384-, or 1536-well plates. Each vial is sufficient for assaying one 96-, 384-, or 1536-well plate.
R8141 (Bulk Kit)	• 10 vials Component A • The entire kit is sufficient for one hundred 96-, 384-, or one hundred fifty 1536-well plates. Each vial is sufficient for assaying ten 96-, 384-well, or fifteen 1536-well plates.
R8143 (Express Kit)	• 2 vials Component A • The entire kit is sufficient for one hundred 96-, 384-, or one hundred fifty 1536-well plates. Each vial is sufficient for assaying fifty 96- or 384-well, or seventy-five 1536-well plates.

Materials Required but Not Provided

Table 2: Reagents and supplies

Item	Suggested Vendor
HBSS Buffer (1X Hank's Balanced Salt solution plus 20 mM HEPES buffer) pH 7.4	• 10X Hank's Balanced Salt Solution (#14065-056, Gibco or equivalent) • 1M HEPES buffer solution (#9319, Irvine Scientific or equivalent) • Water for cell culture (# 9312, Irvine Scientific or equivalent)
Probenecid (inhibitor for the anion-exchange protein) may be required with some cell lines. Prepare a stock solution of 500 mM in 1N NaOH, then dilute to 250 mM in HBSS buffer. Adjust pH of HBSS buffer plus 20mM HEPES to pH 7.4. Prepare loading buffer such that the final in-well concentration of probenecid is 2-2.5 mM.	• Sigma (# P8761) or other chemical suppliers
Assay plates: • 96- or 384-well black-wall, clear bottom plates (assay plates) OR • 1536-well low-base black-wall, clear bottom plates (assay plates) • 1536-well lids	Costar, Nunc, BD or Greiner Greiner #783092 or E&K Scientific #EK16092 Greiner #656191 or E&K Scientific #EK26191
Compound plates: • 96- or 384-well polypropylene plates • 1536-well polystyrene plates	 Costar, Nunc, BD or Greiner Costar, Nunc, BD or Greiner

Storage and Handling

On receipt of the FLIPR Calcium 4 Assay Kit, store contents at –20°C. Under these conditions the reagents are stable for six months in the original packaging.

After formulation, the Loading Buffer is stable for up to eight hours at room temperature. Aliquots can be frozen and stored for up to 5 days without loss of activity.

Quick Start protocol

- Plate cells the previous night in microplates and incubate over night
- The following day prepare the loading buffer
- Remove cell plates from the incubator and add an equal volume of loading buffer to each well (i.e. 25µL of loading buffer to 25µL of cells and media for a 384 well plate)
- Return plates to the incubator and incubate 1h at 37°C
- Prepare compound plates
- Run experiment of FLIPR or FlexStation

Calcium 4 Assay Kit Experimental Protocol

A. Cell Handling

The FLIPR Calcium 4 Assay Kit is designed to work with many cell types, both adherent and non-adherent. We recognize that a variety of cell handling conditions might be adopted at the discretion of the user, based on standard operating procedures in the laboratory. In this section, we provide guidelines on how to prepare the cells for use with the assay kit.

Adherent cells are the most frequently used cells with the kits. They are typically plated the day prior to an experiment and then incubated in a 5% CO_2, 37°C incubator overnight. See Table 3 for suggested plating volumes and seeding densities to create an 80-90% confluent cell monolayer before placing the plates in the FLIPR or FlexStation.

Table 3: Suggested plating volumes and seeding densities

Cell Type (cells/well)	**96-well plate** (100 µL growth medium)	**384-well plate** (25 µL growth medium)	**1536-well plate** (4 µL growth medium)
Adherent cells	20,000 – 80,000	5,000 – 20,000	1,500 – 5,000
Non-adherent cells	40,000 – 200,000	10,000 – 50,000	3,000 – 10,000

For non-adherent cells, we recommend centrifuging cells from culture medium and re-suspending the pellet in culture medium on the day of the experiment. It is recommended after the cells are plated to centrifuge the plates at 100 x *g* for up to 4 minutes (with brake off). Alternatively, non-adherent cells can be treated like adherent cells, plating the day before the assay using the same plating volumes and seeding densities, as long as the cells are seeded onto coated plates (e.g.: poly-D-lysine or collagen) to ensure good attachment to the plate bottom.

B. Preparation of Loading Buffer

The following procedure is designed for preparation of the Loading Buffer per vial of the **Explorer Kit (R8142),** the **Bulk Kit (R8141),** or the **Express Kit (R8143)**.

1. To prepare the 1X HBSS Buffer plus 20mM HEPES for the Bulk and Express Kits only, **(Explorer Kit contains ready to use HBSS buffer plus 20 mM HEPES pH 7.4 in Component B),** pipette 100 mL of 10X Hank's Balanced Salt Solution and 20 mL of 1M Hepes buffer pH 7.4 into 880 mL cell culture treated water.

2. Remove one vial of FLIPR Calcium 4 Assay Reagent **(Component A)** and equilibrate to room temperature.

3. Dissolve contents of Component A vial by adding the appropriate amount of 1X HBSS Buffer plus 20mM HPES as outlined in Table 4. Mix by vortexing (~1-2 min) until contents of vial are dissolved. It is important that contents are *completely* dissolved to ensure reproducibility between experiments.

Table 4: Quantities of 1X HBSS necessary to dissolve Component A contents.

Plate Format	Explorer Kit (R8142)	Bulk Kit (R8141)	Express Kit (R8143)
96- or 384-well	10 mL	10 mL	20 mL
1536-well	6.5 mL	10 mL	20 mL

4. Prepare the Loading Buffer by diluting the Component A vial mixture with an additional volume of 1X HBSS plus 20 mM HEPES Buffer as outlined in Table 5. Multiple washes of the vial are necessary to completely transfer the contents.

Table 5: Quantities of 1X HBSS plus 20 mM HEPES necessary to perform second dilution of Component A.

Plate Format	Explorer Kit (R8142)	Bulk Kit (R8141)	Express Kit (R8143)
96- or 384- well	N/A	90 mL	480 mL
1536-well	N/A	55 mL	300 mL

Note: If your cells require probenecid, then a stock solution should be prepared in 1 N NaOH and added fresh to 1X HBSS buffer plus 20mM HEPES (adjust pH to 7.4 after addition of probenecid) for preparation of the Loading Buffer so that the *final in-well working concentration is 2-2.5 mM*. (Also see procedure on page 4). Do not store frozen aliquots of Loading Buffer with probenecid and always prepare fresh probenecid on the day of the experiment.

Warning: The components supplied are sufficient for proper cell loading. For optimum results it is important **NOT** to add any additional reagents or change volumes and concentrations.

C. Cell Loading using Loading Buffer

1. Remove cell plates from the incubator or centrifuge. Do not remove the supernatant. Add an equal volume of Loading Buffer to each well (100 µL per well for 96-well plates, 25 µL for 384-well plate. ***Note:*** Add 2 µL per well for 1536-well plate by using an AquaMax® DW4 or equivalent device).

   Note: Although Molecular Devices does not recommend washing cells before dye loading growth medium and serum may interfere with certain assays. In this case, the supernatant can be aspirated and replaced with an equal volume of serum-free HBSS plus 20 mM HEPES buffer before adding the Loading Buffer. Alternatively, cells can be grown in serum-free conditions.

2. Incubate cell plates for 1 hour at 37°C and then keep the plates at room temperature until used (loading time should be optimized for your cell line).

   Note: Some assays perform optimally when the plates are incubated at room temperature.

   Warning: Do **NOT** wash the cells after dye loading.

D. Running the Calcium Mobilization Assay

FLIPR

1. After incubation, transfer the plates directly to FLIPR and begin the calcium assay as described in the FLIPR system manual.

2. When performing a signal test prior to an experiment, typical average baseline counts range from 8,000–12,000 RFU (FLIPR 1, FLIPR^{384} or FLIPR^{3}) or 800–1,200 RFU on FLIPR$^{TETRA®}$.

3. Suggested experimental setup parameters for each FLIPR system are as follows.

   Fast addition speeds close to the cell monolayer are recommended to ensure better mixing of compounds and lower signal variance across the plate. However, further assay development, adjustment of the volume, height and speed of dispense, is recommended to optimize your cell response.

Table 6. Experimental setup parameters for FLIPR 1, FLIPR^{384} & FLIPR^{3}

Parameters	**96-well plate FLIPR 1, FLIPR^{384}**	**384-well plate FLIPR^{384}**	**384-well plate FLIPR^{3}**
Exposure (sec)	0.4	0.4	0.4
Camera Gain	N/A	N/A	50-80
Addition Volume (µL)	50	12.5	12.5
Addition Height (µL)	210-230	35-45	35-45
Compound Concentration (Fold)	5X	5X	5X
Addition Speed (µL/sec) Adherent Cells	50-100	10-20	25-40
Addition Speed (µL/sec) Non adherent Cells	10-20	5-10	10-25

Table 7. Experimental setup parameters for FLIPR^{TETRA}

Parameters	96-well plate	384-well plate	1536-well plate
Exposure (sec)	0.4	0.4	0.4
Camera Gain	50-130	50-130	50-130
Addition Volume (µL)	50	12.5	1
Compound Concentration (Fold)	5X	5X	7X
Excitation LED (nm)	470-495	470-495	470-495
Emission Filter (nm)	515-575	515-575	515-575
Intensity (%)	80	80	80
Addition Height (µL)	210-230	35-45	2
Tip Up Speed (mm/sec)	10	10	5
Addition Speed Adherent Cells (µL/sec)	50-100	30-40	4-7
Addition Speed Non Adherent Cells (µL /sec)	10-20	10-20	1-5

FlexStation

1. Recommended experimental setup parameters for the FlexStation are as follows. Set up your FlexStation using SOFTmax Pro® before you read the plate.

Table 8. Experimental setup parameters for 96- and 384-well plates on FlexStation

Fluorescence Parameters	96-well	384-well
Excitation Wavelength (nm)	485	485
Emission Wavelength (nm)	525	525
Emission Cut-Off (nm)	515	515

Parameters	96-well	384-well
PMT Sensitivity	6	6
Pipette Height (µL)	230	50
Transfer Volume (µL)	50	12.5
Compound Concentration (Fold)	5X	5X
Addition Speed (Rate) Adherent Cells	2	2-3
Addition Speed (Rate) Non Adherent Cells	1	1

2. After incubation (see notes in FLIPR section), transfer the assay plate directly to the FlexStation assay plate carriage and run the assay.

3. The calcium flux peak should be complete within 1 to 3 min after addition. For an entire plate however, the plate will not be complete until all chosen columns are finished. We recommend collecting data for a minimum of 6 min during assay development, for a single column to determine appropriate assay time prior to running the entire plate.

4. Analyze the data using SOFTmax Pro.

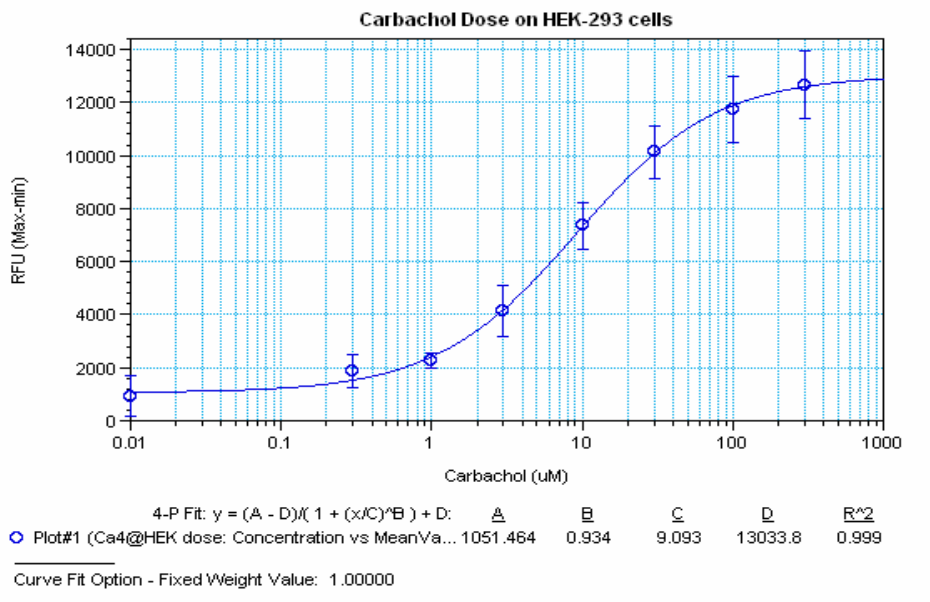

Figure 1. Carbachol dose response in HEK-293 cells. Cells were seeded overnight at 25 µL per well in a 384-well Poly-D-Lysine coated plate. Cells were incubated with 25 µL of Calcium 4 Assay Kit for 1 h at 37°C. Carbachol was added (12.5 µL /well) to achieve the final indicated concentration (n = 24).

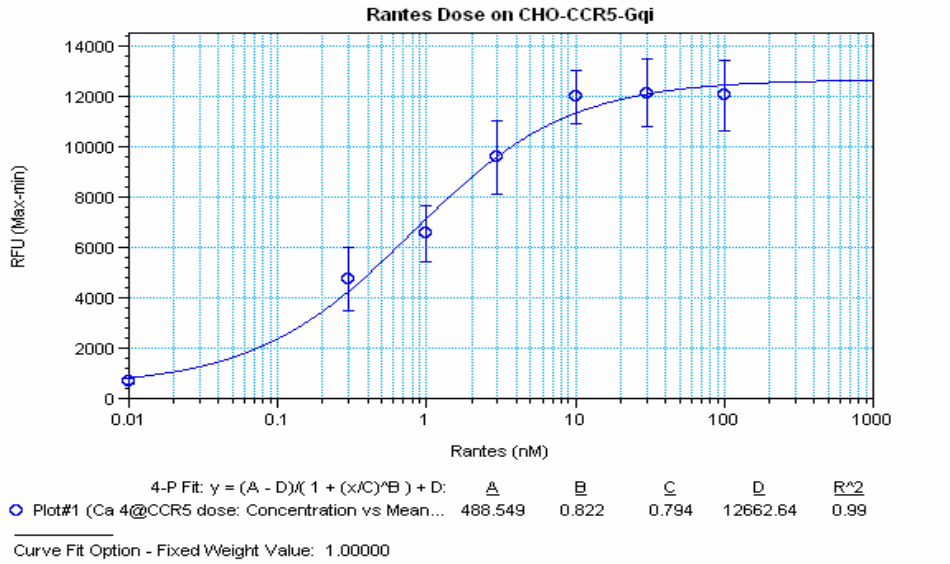

Figure 2. CHO cells stably transfected with CCR5 and Gqi5 were seeded overnight at 25 µL per well in a 384-well costar plate. Cells were incubated with 25 µL of Calcium 4 Assay Kit for 1 h at 37°C. RANTES was added (12.5 µL /well) to achieve the final indicated concentration (n = 24).

Trouble-shooting Guide

Fluorescence drop upon compound addition ("dip")
This may be the result of dislodging cells from the well bottom during addition. Lowering the addition/dispense speed or adjusting addition height or both should solve the problem in this case.

Another potential reason is the dilution of the non-fluorescent compound into a plate with media containing fluorescent components (like DMEM media). This Ca4 kit mediates this issue compared to earlier developed Ca-kits.

Adding volumes greater than recommended may increase the initial fluorescence drop. In these cases it may be necessary to adjust the volumes of the components. The recommended volume of the Loading Buffer is 100 μL for 96-well plates, 25 μL for 384-well plates and 2 μL for 1536-well plates.

Warning: Decreasing the final in-well concentration of the Loading Buffer may decrease the response of the assay. If only one addition is required, then adding a higher concentration of compound in low volume could help reduce any fluorescence drop upon addition.

Serum-sensitive cells or targets
Some cells are serum-sensitive resulting in oscillations of intracellular calcium that could interfere with results. Also, some target receptors or test compounds may interact with serum factors. In these cases, serum-containing growth medium should be removed prior to addition of loading buffer. The volume of growth medium removed should be replaced with an equal volume of 1X HBSS plus 20mM HEPES buffer before loading. Alternatively cells could be incubated overnight in lower concentrations of FBS and not washed prior to the addition of Dye Loading Buffer.

Cells tested with buffer plus DMSO show a calcium response.
Buffer used for the negative control wells should contain the same final concentration of DMSO as is present in the wells containing the test compounds. However, this concentration of DMSO could cause a calcium flux. In these cases, add DMSO to the Loading Buffer such that the final concentration of DMSO in the wells does not change after buffer addition.

Precipitation in the Reagent Buffer.
The FLIPR Calcium 4 Assay Kit is compatible with numerous buffers. Use buffers shown to work in previously established assays, if available.

Response is smaller than expected.
Agonists and antagonists may stick to the tips and trays. Use 0.1% BSA in all compound buffer diluents and presoak tips in compound buffer containing 0.1% BSA. (***Note:*** Do not use the same compound plate for presoaking and compound addition when using a 384 Pipettor head in the FLIPR System. Instead, use a 'Boat' for the presoak.)

Apparent well-to-well variation is observed.
AquaMax DW4 or equivalent dispenser is recommended for use with all additions off the FLIPR or FlexStation if apparent well-to-well variation is observed. In some cases allowing the plates to stand at room temp prior to use in the assay may decrease well-to-well variation.

Product Use Limitations and Warranty

All Molecular Devices reagent products are sold For Research Use Only. Reagents may contain chemicals that are harmful. Due care should be exercised to prevent direct human contact with the reagent.

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