FLIPR® Calcium 5 Assay Kit

The FLIPR Calcium 5 Assay Kit from Molecular Devices® uses a unique fluorophore that exhibits a large signal window, and is amenable to incubation at 37°C or room temperature, facilitating fully-automated screens that can run overnight.

Table 1-1: Available Kits

Assay Kit	Explorer Kit	Bulk Kit	Express Kit
FLIPR® Calcium 5 Assay Kit	R8185	R8186	R8187

Product manufactured by Molecular Devices, LLC.
3860 N. First Street, San Jose, California, 95134, United States of America.
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Chapter 1: About the FLIPR Calcium 5 Assay Kit

Calcium assays from Molecular Devices employ sensitive calcium indicators and masking dyes. The FLIPR Calcium 5 Assay Kit contains a new formulation that further enhances the calcium flux assay with an increased signal window. Kit components are mixed with buffer and incubated for approximately one hour with cells. During incubation, the indicator passes through the cell membrane and esterases in the cytoplasm cleave the AM portion of the molecule. Some cell lines have an anion-exchange protein that requires the use of an anion reuptake inhibitor such as probenecid to retain the calcium indicator. After incubation, the cells are ready to be assayed. The masking dye does not enter the cell, but significantly reduces background originating from residual extracellular fluorescence of calcium indicator, media and other components. Once the target is activated, direct measurement of intracellular fluorescence change due to increased calcium concentration is enabled.

Assay Principle

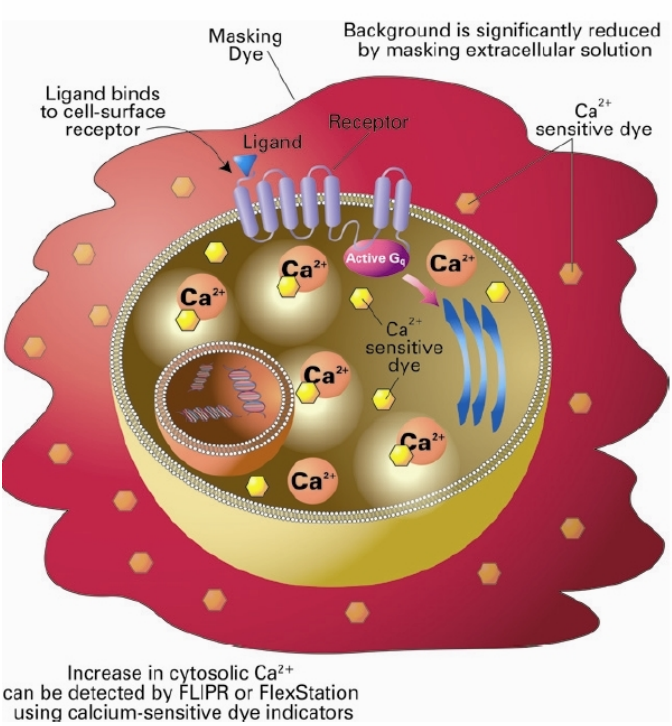


Figure 1-1: Calcium 5 Assay Principle

Advantages

Conventional calcium mobilization protocols are multi-step procedures, which begin by pre-washing the cells, loading them with a calcium indicator such as Fluo-3 or Fluo-4, followed by extensive cell washing before 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 Assays to eliminate the cause of data variability and reduce the number of steps in the conventional wash protocol using Fluo-3 or Fluo-4. Building on this experience, the FLIPR Calcium 5 Assay Kit uses the same proven quench technology as the FLIPR Calcium 4 Assay Kit in combination with a novel calcium indicator. This homogenous approach introduces the following improvements over previous kits as well as conventional wash protocol:

- 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-well, 384-well, or 1536-well formats

Application

The FLIPR Calcium 5 Assay Kit uses a newly improved formula that further enhances the signal window of the assay and makes difficult assays more amenable to high-throughput screening. The kit provides a homogeneous assay designed to work for the majority of GPCRs, including chemokine and other difficult receptors, sticky compounds, allosteric modulators, as well as for calcium channels.

Chapter 2: Materials and Equipment

Kit Components

FLIPR Calcium 5 Assay Kit

Table 2-1: Components of the FLIPR Calcium 5 Assay Kit

Item	Explorer Kit (R8185)	Bulk Kit (R8186)	Express Kit (R8187)
Component A	10 vials	10 vials	2 vials
Assay Buffer (Component B) 20 mM HEPES buffer + 1X Hank's Balanced Salt solution (HBSS), pH 7.4	1 bottle	—	—

- The entire Explorer Kit is sufficient for ten (10) 96-well, 384-well, or 1536-well microplates. Each vial is sufficient for one (1) microplate, depending on dead volume of the dispenser used.
- The entire Bulk Kit is sufficient for one-hundred (100) 96-well or 384-well microplates, or one hundred fifty (150) 1536-well microplates. Each vial is sufficient for ten (10) 96-well or 384-well microplates, or fifteen (15) 1536-well microplates, depending on dead volume of the dispenser used.
- The entire Express Kit is sufficient for one hundred (100) 96-well or 384-well, or one hundred fifty (150) 1536-well microplates. Each vial is sufficient for assaying fifty (50) 96-well or 384-well microplates, or seventy five (75) 1536-well microplates, depending on dead volume of the dispenser used.

Materials Required But Not Provided

Table 2-2: Reagents and Supplies

Item	Suggested Vendor
Component B*: 20 mM HEPES buffer + 1X Hank's Balanced Salt solution (HBSS), pH 7.4 *Component B is provided for Explorer kits only.	10X Hank's Balanced Salt Solution (Gibco #14065-056, or equivalent) 1M HEPES buffer solution (Irvine Scientific #9319, or equivalent) Water for cell culture (Irvine Scientific #9312, or equivalent)
FLIPR® validated pipette tips, 96, 384 or 1536 gasket.	Molecular Devices
Probenecid, an inhibitor for the anion-exchange protein, might be required for some cell lines to ensure that the dye stays inside the cell and is not pumped back out. Prepare a stock solution of 500 mM in 1N NaOH, and then dilute to 250 mM in HBSS buffer. Prepare Loading Buffer such that the final in-well concentration of probenecid is 2.5 mM when added to cells.	Sigma #P8761, or other chemical suppliers **Tip:** Use of water-soluble probenecid is also possible following individual manufacturer instructions.

Table 2-2: Reagents and Supplies (continued)

Item	Suggested Vendor
Assay Microplates: 96-well or 384-well black-wall, clear bottom assay plates 1536-well low-base, black wall, clear bottom assay plates 1536-well plate lids	 Costar, Nunc, BD or Greiner Greiner 783092 or equivalent Greiner 656191 or equivalent
Compound Microplates: 96-well or 384-well polypropylene plates 1536-well polypropylene plates	 Costar, Nunc, BD or Greiner Costar, Nunc, BD or Greiner

Storage and Handling

On receipt of the FLIPR Calcium 5 Assay Kit, store contents at –20°C (-4°F), and store other components at 4°C (39.2°F). Under these conditions, the reagents are stable for six (6) months in the original packaging.

After reconstitution, the Loading Buffer is stable for up to eight (8) hours at room temperature. Aliquots can be frozen and stored (without probenecid) for up to one (1) week without loss of activity.

WARNING! Reagents can contain chemicals that are harmful. Exercise care when handling reagents as described in the related safety data sheet (SDS or MSDS). The safety data sheet is available in the Knowledge Base on the Molecular Devices support web site: www.moleculardevices.com/service-support

Compatible Molecular Devices Instruments

The FLIPR Calcium 5 Assay Kit is designed to be used with the following Molecular Devices instruments and their corresponding tips:

- FLIPR Penta High-Throughput Cellular Screening System
- FLIPR® Tetra High-Throughput Cellular Screening System
- FlexStation 3 Multi-Mode Microplate Reader

Chapter 3: Experimental Protocol

Quick Start Protocol

To run a FLIPR Calcium 5 Assay Kit protocol:

1. Plate cells in microplates and incubate overnight at 37°C (98.6°F), 5% CO_2.
2. Prepare the Loading Buffer the following day.
3. Remove cell plates from the incubator and add an equal volume of loading buffer to each well.
   For example, for a 384-well microplate, add 25 µL of Loading Buffer to 25 µL of cells and media.
4. Return prepared plates to the incubator and incubate one hour at 37°C (98.6°F), 5% CO_2.
5. Prepare compound plates.
6. Run the experiment on a FLIPR instrument or FlexStation 3 instrument.

Cell Handling

The FLIPR Calcium 5 Assay Kit is designed to work with many cell types, both adherent and non-adherent. Standard procedures vary across laboratories and we recognize that a variety of cell handling conditions might be adopted at the discretion of the user. In this section, we provide general guidelines for preparing 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 (98.6°F) incubator overnight. See Table 3-1 for suggested plating volumes and seeding densities to create an 80-90% confluent cell monolayer before placing the plates in a FLIPR instrument or FlexStation 3 instrument.

Table 3-1: Suggested Plating Volumes and Seeding Densities

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

For non-adherent cells, we recommend centrifuging cells from culture medium and re-suspending the pellet in culture medium or appropriate buffer of choice on the day of the experiment. Cells can be dye-loaded in a tube or while plated. After the cells are plated, 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, such as poly-D-lysine or collagen, to ensure good attachment to the plate bottom.

Preparing Loading Buffer

The following procedure is designed for preparation of the FLIPR Calcium 5 Assay Kit Loading Buffer per vial of the Explorer Kit. More volumes for the Bulk Kit and Express Assay Kit are included in Table 3-2.

To prepare loading buffer:

1. Remove one vial of Component A from the freezer and equilibrate to room temperature.
2. Equilibrate Component B to room temperature.
   You can substitute 1X HBSS Buffer plus 20 mM HEPES for Component B.
3. Dissolve the contents of one Component A vial by adding 10 mL of Component B and then mix by vortexing for ~1 to 2 minutes until the contents of the vial are dissolved.

   Note: It is important that the contents are completely dissolved to ensure reproducibility between experiments.

4. Prepare the Loading Buffer by diluting the Component A vial mixture with an additional volume of Component B. Multiple washes of the vial are necessary to completely transfer the contents.

Table 3-2: Required volumes to formulate FLIPR Calcium 5 Assay Kits

Plate Well-Type	Volumes to Formulate FLIPR Calcium 5 Assay Kit	Explorer Kit (R8190)	Bulk Kit (R8191)	Express Kit (R8195)
96 or 384	Volume to dissolve Component A	10 mL	10 mL	20 mL
	More required for correct volume	None	90 mL	480 mL
1536	Volume to dissolve Component A	6.5 mL	10 mL	25 mL
	More required for correct volume	None	55 mL	300 mL

Note: If the cells require probenecid (such as CHO or other cells containing an organic anion transporter), then a 500 mM stock solution should be prepared by adding 1 N NaOH in tissue culture treated water, vortexing and diluting to 250 mM with 1X HBSS buffer plus 20 mM HEPES. Prepare the Loading Buffer so that the final in-well working concentration of probenecid is 2.5 mM. Adjust Loading Buffer pH to 7.4 after addition of probenecid. See the procedure for making probenecid in Materials Required But Not Provided on page 5. Assay development might be required to determine the best concentration.

CAUTION! Do not store frozen aliquots of Loading Buffer with probenecid, and always prepare new probenecid on the day of the experiment. Use of water-soluble probenecid is also possible following individual manufacturer instructions.

CAUTION! The components supplied are sufficient for proper cell loading. For optimal results it is important NOT to add other reagents or to change volumes and concentrations.

Loading Cells Using Loading Buffer

To load cells using loading buffer:

1. Remove cell plates from the incubator or centrifuge.

   CAUTION! Do not remove the supernatant.

2. Add an equal volume of Loading Buffer to each well:
   - 96-well plates—add 100 µL per well
   - 384-well plates—add 25 µL per well
   - 1536-well plates—add 2 µL dye per well by using an appropriate liquid handling device for cells

   Note: Molecular Devices does not recommend washing cells before dye loading because growth medium and serum might 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. As an alternative, cells can be grown in reduced serum or serum-free conditions.

3. After adding dye, incubate cell plates for 1 hour at 37°C with 5% CO_2 and then keep the prepared plates at room temperature until used. The loading time should be optimized for each cell line and target.

   Note: Some assays do optimally when the microplates are incubated at room temperature or for different loading times.

   CAUTION! Do not wash the cells after dye loading.

Running the Calcium Mobilization Assay on a FLIPR Instrument

After incubation, transfer the microplates to the FLIPR instrument and start the calcium assay as described in the user guide for the instrument.

Do the assay signal test before the experiment. The LED intensity and the gate or gain can be adjusted to get the desired RFU range. It is important to keep the exposure time short.

- For a FLIPR Tetra System or FLIPR Penta instrument with an EMCCD camera, adjust typical average baseline counts to a range from 800 RFU to 1500 RFU for EACH LED set. Keep the gain setting the same for the two LEDs.
- For a FLIPR Penta instrument with an HS EMCCD camera, adjust typical average baseline counts to a range from 5000 RFU to 10,000 RFU.
- For a FLIPR Tetra System or FLIPR Penta instrument with an ICCD camera, adjust baseline counts to a range from 6000 RFU to 9000 RFU.

Update the assay signal test to transfer the adjusted settings to the protocol.

Suggested experimental setup parameters for each FLIPR instrument are listed in the following tables.

Faster addition speeds closer to the cell monolayer are recommended to ensure better mixing of compounds and lower signal variance across the plate. Molecular Devices recommends further assay development and adjustment of the volume, height, and speed of dispense to optimize the individual cell response.

Recommended Settings for the FLIPR Penta Instrument

Before reading the microplate, set up your FLIPR system using the following recommended experiment settings. Your settings depend on the camera installed in your instrument.

Recommended EMCCD Camera Parameters

Table 3-3: Experimental setup parameters for FLIPR Tetra System and FLIPR Penta system with EMCCD camera

Parameter	96-Well Plate	384-Well Plate	1536-Well Plate
Exposure (seconds)	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
LED Intensity (%)	80	80	80
Addition Height (µL)	210-230	35-45	2
Tip Up Speed (mm/sec)	10	10	5
Addition Speed (µL/sec) Adherent Cells	50-100	30-40	4-7
Addition Speed (µL/sec) Non-Adherent Cells	10-20	10-20	1-5

Recommended HS EMCCD Camera Parameters

The following settings are for use in Normal camera mode.

Table 3-4: Experimental setup parameters for FLIPR Penta system with HS EMCCD camera

Parameter	96-Well Plate	384-Well Plate	1536-Well Plate
Exposure (seconds)	0.1	0.1	0.1
Read Interval (s)	1	1	1
Camera Gain	4	6.5	4
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
LED Intensity (%)	50	50	50
Addition Height (µL)	210–230	30	1
Tip Up Speed (mm/sec)	10	10	10

Table 3-4: Experimental setup parameters for FLIPR Penta system with HS EMCCD camera (continued)

Parameter	96-Well Plate	384-Well Plate	1536-Well Plate
Addition Speed (µL/sec) Adherent Cells	50	30	8
Addition Speed (µL/sec) Non Adherent Cells	20	20	2

Note: Contact Molecular Devices for HS EMCCD camera upgrade options.

Recommended ICCD Camera Parameters

Table 3-5: Experimental setup parameters for FLIPR Tetra System and FLIPR Penta system with ICCD camera

Parameter	96-Well Plate	384-Well Plate	1536-Well Plate
Exposure (seconds)	0.53	0.53	0.53
Camera Gain	Fixed at 2,000	Fixed at 2,000	Fixed at 2,000
Camera Gate	6%	6%	6%
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
LED Intensity (%)	50	50	50
Addition Height (µL)	210-230	35-45	2
Tip Up Speed (mm/sec)	10	10	5
Addition Speed (µL/sec) Adherent Cells	50-100	30-40	4-7
Addition Speed (µL/sec) Non Adherent Cells	10-20	10-20	1-5

Running the FLIPR Calcium 5 Assay on a FlexStation 3 Instrument

Before reading the microplate, set up the FlexStation 3 instrument using a SoftMax® Pro Software protocol. Use the recommended parameters listed in Table 1.

Molecular Devices recommends that parameters be optimized for each cell line and targeted to deliver the best performance for your assay.

After incubation, transfer the microplates directly to the FlexStation 3 reader plate carriage and run the assay.

In an individual well or column of wells, the peak(s) should be complete within 1 to 3 minutes after addition. For an entire plate, however, the protocol will not complete until all chosen columns are finished. The assays are run one column at a time.

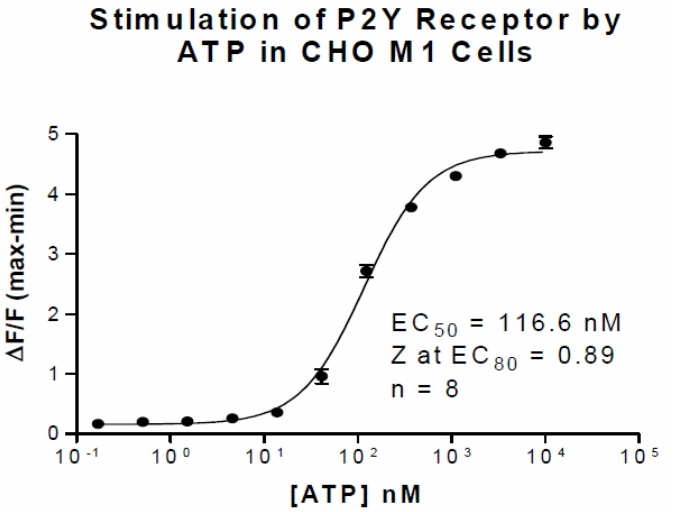
Analyze the data using the SoftMax Pro Software.

Recommended Settings for the FlexStation 3 Instrument

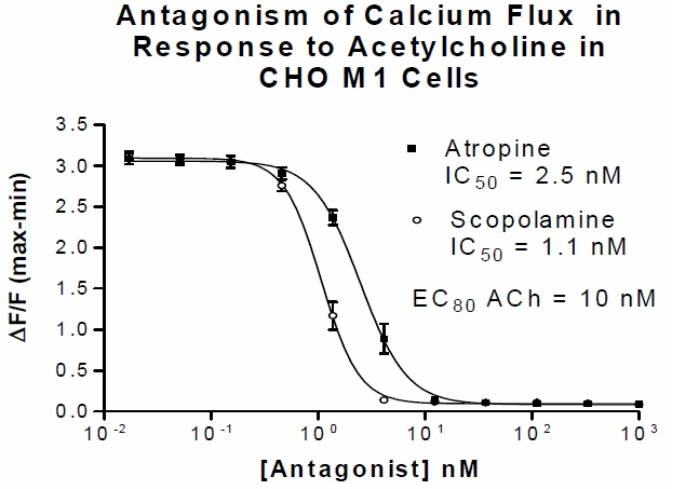
Table 3-6: Experiment Setup Parameters for the FlexStation 3 Instrument

Parameter	96-well microplate	384-well microplate
Read Mode	Fluorescence	Fluorescence
Read Type	Flex	Flex
Excitation Wavelength (nm)	485	485
Emission Wavelength (nm)	525	525
Automatic Emission Cut-Off (nm)	515	515
PMT Gain	High	High
Flashes per Read	6	6
Pipette Height (µL)	230	40
Transfer Volume (µL)	50	12.5
Compound Concentration (Fold)	5X	5X
Addition Speed (Rate)	Adherent Cells: 3 Non-Adherent Cells: 1	Adherent Cells: 2 to 3 Non-Adherent Cells: 1

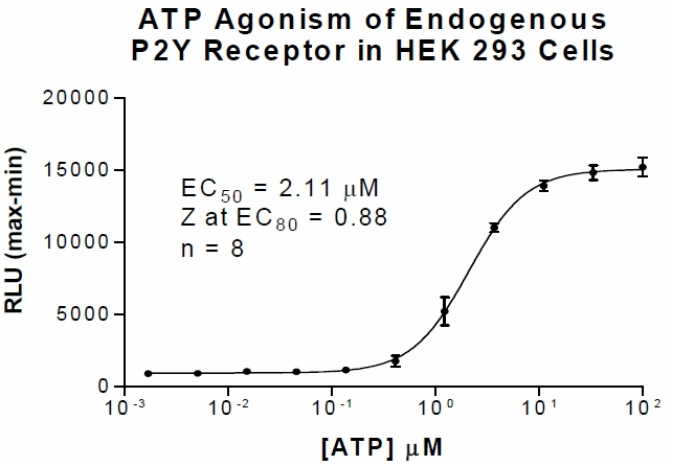
Data Analysis Examples



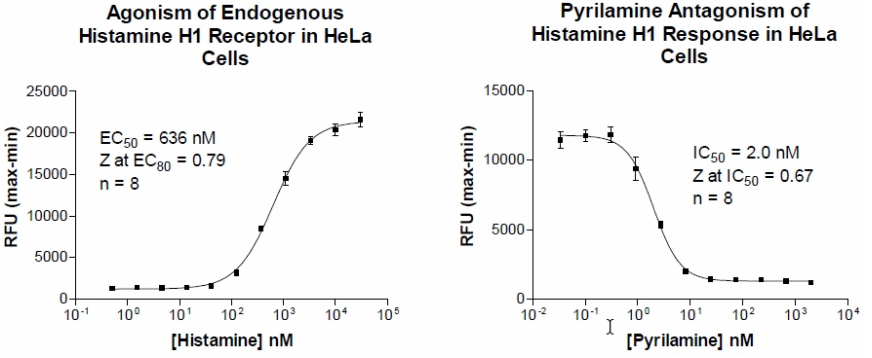
ATP dose response in CHO M1 cells. Cells were seeded overnight at 25 µL per well in a 384-well black wall clear bottom-plate. Cells were incubated with 25 µL of Calcium 5 Kit including probenecid for 45 minutes at 37°C 5% CO_2 followed by 15 minutes at room temperature. 12.5 µL 5X ATP was added per well during detection on a FLIPR Tetra instrument with ICCD camera to achieve the final indicated concentration.



CHO M1 cells were seeded overnight at 25 µL per well in a 384-well black wall clear bottom plate. Cells were incubated with 25 µL of Calcium 5 Assay including probenecid for 45 minutes at 37°C 5% CO_2 followed by 15 minutes at room temperature. 12.5 uL 5X antagonist was added followed by a 15 minute incubation at room temperature. A final concentration of 10 nM acetylcholine was added as challenge agonist during detection on the FLIPR Tetra instrument with EMCCD camera.



ATP 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 5 Assay for 45 minutes at 37°C 5% CO_2 followed by 15 minutes at room temperature. ATP was added (12.5 µL /well) to achieve the final indicated concentration on the FLIPR Tetra instrument with ICCD camera.



HeLa cells with endogenous Histamine H1 receptor were seeded overnight at 25 µL per well in a 384-well black clear-bottom plate. Cells were incubated with 25 µL of Calcium 5 Assay including probenecid for 45 minutes at 37°C 5% CO_2 followed by 15 minutes at room temperature. 4a. For the agonist experiment, a 5X dose response of histamine was added (12.5 µL /well) to achieve the final indicated concentrations. 4b. For the antagonist experiment, a 5X dose of pyrilamine (12.5 µL /well) was added followed by a 15 minute incubation at room temperature to achieve the indicated concentrations. EC_{80} concentration of histamine was added as challenge agonist during detection on the FLIPR Tetra instrument with ICCD camera.

Chapter 4: Troubleshooting

This section provides solutions to problems that you might experience when running calcium flux assays.

Note: Performance of the Molecular Devices Reagent Kits on Molecular Devices instruments have been validated for use with Molecular Devices pipette tips.

Fluorescence Drop Upon Compound Addition

This can be the result of dislodging cells from the well bottom during addition. Lowering the dispense speed or adjusting the addition height or both should solve the problem.

Another potential reason is the dilution of the non-fluorescent compound into a plate with media containing fluorescent components, like DMEM media. This Calcium kit mitigates this issue compared to earlier developed Calcium kits. See Assays.

Adding volumes greater than recommended can increase the initial fluorescence drop. In these cases it might 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.

CAUTION! Decreasing the final in-well concentration of the Loading Buffer can 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 20 mM 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 with 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 Assay Kits are compatible with numerous buffers. Use buffers shown to work in previously established assays, if available.

Apparent Well-to-Well Variation is Observed

An automated liquid dispenser is recommended for use with additions of cells or dye prior to the assay if apparent well-to-well variation is observed. In some cases allowing the plates to stand at room temperature prior to use or adding a single mix cycle in the compound or assay plate can decrease well-to-well variation.

Response is Smaller than Expected

The agonist and antagonist may stick to the tips and trays. Use up to 1% BSA in all compound buffer diluents and pre-soak tips in compound buffer with up to 1% BSA.

Note: Do not use the same source plate for pre-soaking and compound addition when using a 384 Pipettor head. Instead, use a 'Boat' for the pre-soak.

Obtaining Support

Molecular Devices is a leading worldwide manufacturer and distributor of analytical instrumentation, software, and reagents. We are committed to the quality of our products and to fully supporting our customers with the highest level of technical service.

Our Support website, www.moleculardevices.com/service-support, has a link to the Knowledge Base, which contains technical notes, software upgrades, safety data sheets, and other resources. If you still need assistance after consulting the Knowledge Base, you can submit a request to Molecular Devices Technical Support.

Please have the instrument serial number (on the rear of the instrument), or reagent kit lot number, and any related sample data files available when you call.

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