# FLIPR® Calcium Evaluation Assay Kit

The Calcium Evaluation Assay Kit provides a convenient way to compare the performance of FLIPR® Calcium 4, Calcium 5, and Calcium 6 Kits from Molecular Devices side by side and determine the optimal kit for assay conditions, targets, or cell lines. Each kit provides a mix-and-read procedure in which cells are incubated with the reagents for one to two hours and transferred directly to FLIPR® or FlexStation® Multi Mode Microplate Reader for evaluation. There are no intermediate wash-steps involved. Based on Molecular Devices proprietary masking dye technology, the kits provide a simple and reliable fluorescence-based assay for detecting changes in intracellular calcium. The Calcium 4 and 5 Kits share the same quench technology, but have two different calcium indicators.

Patents: http://www.moleculardevices.com/patents

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# Chapter 1: Introduction

Calcium assays from Molecular Devices employ sensitive calcium indicators and masking dyes. The evaluation kit provides the opportunity to determine which combination of calcium indicator and quench technology will provide optimal response depending on assay conditions. The Calcium 5 Assay contains a new calcium indicator; however, it has the same quench technology that's used in the Calcium 4 Assay. This combination 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 where 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

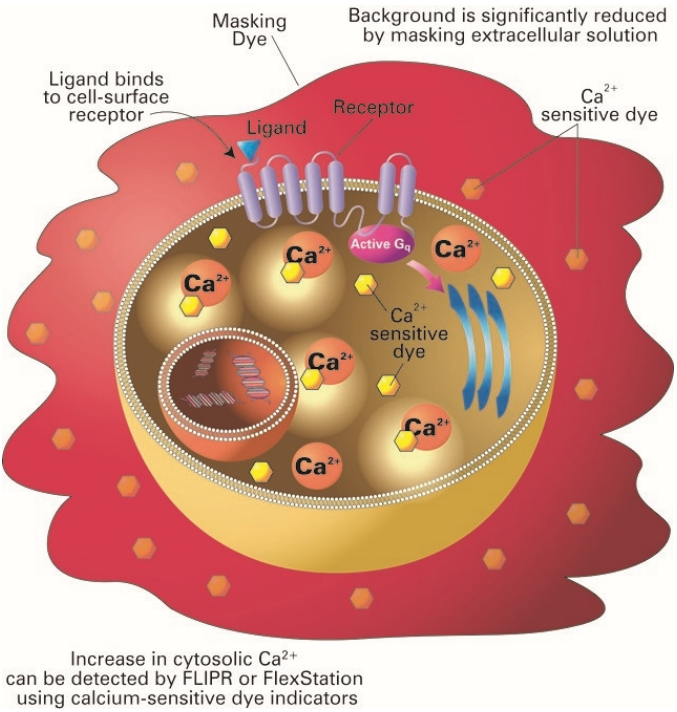


**Figure 1-1: Calcium Assay Principle**

## 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 introduce 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. Calcium 6 Assay Kit uses a newly improved calcium dye formula that further enhances the signal window of the assay and makes difficult assays more amenable to high-throughput screening. Calcium 5 kit uses the proven Calcium 4 quench technology in combination with a novel calcium indicator. The homogenous approach of all 3 kits introduces the following improvements over other kits as well as the 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-, 384-, or 1536-well formats

The Calcium Evaluation Kit includes 3 vials each of Calcium 4, Calcium 5 and Calcium 6 dye with corresponding quench technology. Identification of the optimal detection system for each assay will result in the following improvements for most cell systems:

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

## Applications

The kit provides three different homogeneous assays for calcium flux. It is designed to help you evaluate which of the FLIPR Kits will work best with your target, whether it be GPCR or calcium channel.

# Chapter 2: Materials and Equipment

## Kit Components

### Table 2-1: FLIPR Calcium Evaluation Assay Kit (p/N R1872) Contents

| Reagent | Description |
|---|---|
| R8172 (Evaluation Kit) | • 3 vials Component A of the Calcium 4 kit<br>• 3 vials Component A of the Calcium 5 kit<br>• 3 vials Component A of the Calcium 6 kit<br>• 1 bottle Component B 1X Hank's Balanced Salt solution (HBSS) plus 20 mM HEPES buffer, pH 7.4<br>• Each vial is sufficient for assaying one 96-, 384-, or 1536-well plate. |

## Materials Required But Not Supplied

### Table 2-2: Reagents and Supplies

| Item | Suggested Vendor |
|---|---|
| **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 plus 20mM HEPES buffer. Prepare loading buffer such that the final in-well concentration of probenecid is 2.5 mM after adding to cells. | Sigma (# P8761) or other chemical suppliers |
| **Assay plates:**<br>• 96- or 384-well black-wall, clear bottom plates (assay plates) OR<br>• 1536-well low-base black-wall, clear bottom plates (assay plates)<br>• 1536-well lids | Costar, Nunc, BD or Greiner<br>Greiner #783092 or E&K Scientific #EK16092<br>Greiner #656191 or E&K Scientific #EK26191 |
| **Compound plates:**<br>• 96- or 384-well polypropylene plates<br>• 1536-well polystyrene plates | Costar, Nunc, BD or Greiner<br>Costar, Nunc, BD or Greiner |

## Storage and Handling

On receipt of the FLIPR Calcium Evaluation 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.

# Chapter 3: Calcium Evaluation Assay Kit Experimental Protocol

## Quick Start Protocol

- Plate cells the previous night in microplates and incubate over night
- The following day prepare the Loading Buffer for each of the Calcium kits.
- Remove cell plates from the incubator and add an equal volume of the different loading buffers 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 for 1h at 37°C for Calcium 4 and Calcium 5, 2h at 37°C for Calcium 6.
- Prepare compound plates
- Run experiment on FLIPR or FlexStation instrument

## Cell Handling

The FLIPR Calcium Evaluation Assay Kit is designed to identify the optimal Molecular Devices Calcium kit for your assay of choice. 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 kits. For optimal comparison of the kits, we recommend running them side-by-side on the same plate.

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 instruments.

**Table 3-1: 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 – 30,000 | 1,500 – 5,000 |
| Non-adherent Cells | 40,000 – 200,000 | 10,000 – 60,000 | 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.

## Preparation of Loading Buffer

The following procedure is designed for preparation of the Loading Buffer per 1 vial each of the Evaluation Kit (R8172).

1. Remove one vial of Component A each (Calcium 4, Calcium 5, Calcium 6) from the FLIPR Calcium Evaluation kit and equilibrate to room temperature.
2. Dissolve contents of Component A vials by adding the appropriate amount of Component B (1X HBSS Buffer plus 20 mM HEPES, pH 7.4) to each as outlined in Table 4. Mix by vortexing (~1-2 min) until contents of vials are dissolved. It is important that contents are *completely* dissolved to ensure reproducibility between experiments.

**Table 3-2: Quantities if 1X HBSS Necessary to Dissolve Compound A Contents**

| Plate Format | Evaluation Kit (R8172)<br>Explorer Kit Sized Vials |
|---|---|
| 96-well or 384-well | 10 mL |
| 1536-well | 6.5 mL |

**Note:** If your cells require probenecid, then a 500 mM stock solution should be prepared by adding 1N NaOH, 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 is 2.5 mM*. Adjust Loading Buffer pH to 7.4 after addition of probenecid. Refer to the procedure for making probenecid on page 4. Do not store frozen aliquots of Loading Buffer with probenecid and always prepare fresh probenecid on the day of the experiment. Water soluble probenecid may also be used following supplier instructions.

**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.**

## Cell Loading Using 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 a cell dispensing 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 low serum or 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.**

# Running the Calcium Mobilization Assay - Instrument Settings

## FLIPR Instrument Settings

1. After incubation, transfer the plates directly to FLIPR read position and begin the calcium assay as described in the system manual.
2. When performing a signal test prior to an experiment, typical average baseline counts range from 7,000 – 12,000 RFU (FLIPR 1, FLIPR$^{384}$ or FLIPR$^{3}$) or 700 – 1,200 RFU on the FLIPR$^{TETRA®}$ system with EMCCD camera, or 5,000 - 7,000 RFU on the FLIPR$^{TETRA®}$ system with ICCD camera.
3. Suggested experimental setup parameters for each FLIPR system are as follows:
   Faster 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 3-3: Experimental Setup Parameters for FLIPR$^{1}$, FLIPR$^{384}$, & FLIPR$^{3}$**

| Parameter | 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 Adherent Cells (µL/sec) | 50-100 | 10-20 | 25-40 |
| Addition Speed Non-adherent Cells (µL/sec) | 10-20 | 5-10 | 1-25 |

### Table 3-4: Experimental Setup Parameters for FLIPR$^{TETRA}$ 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 |
| 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 |

### Table 3-5: Experimental Setup Parameters for FLIPR$^{TETRA}$ 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 Adherent Cells (µL/sec) | 50-100 | 30-40 | 4-7 |
| Addition Speed Non-adherent Cells (µL/sec) | 10-20 | 10-20 | 1-5 |

### Table 3-6: Experimental Setup Parameters for 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 |

The following settings are for use in Normal camera mode.

### Table 3-7: 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 |
| Addition Speed (µL/sec) Adherent Cells | 50 | 30 | 8 |
| Addition Speed (µL/sec) Non Adherent Cells | 20 | 20 | 2 |

## FlexStation Instrument Settings

1. Recommended experimental setup parameters for the FlexStation instrument are as follows. Set up your FlexStation instrument using SoftMax® Pro Data Acquisition and Analysis Software before you read the plate.

**Table 3-8: Experimental Setup Parameters for 96-well and 384-well Plates on FlexStation Instrument**

| Fluorescence Parameter | 96-well Plate | 384-well Plate |
|---|---|---|
| Excitation Wavelength (nm) | 485 | 485 |
| Emission Wavelength (nm) | 525 | 525 |
| Auto Emission Cut-Off (nm) | 515 | 515 |

**Table 3-9: Software Parameters**

| Parameter | 96-well Plate | 384-well Plate |
|---|---|---|
| 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 instrument section), transfer the assay plate directly to the FlexStation instrument assay plate carriage and run the assay.
3. The calcium flux signal 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 for a single column during assay development to determine appropriate assay time prior to running the entire plate. Adjust the time accordingly.
4. Analyze the data using SoftMax Pro Software.

FLIPR Calcium Assay Examples

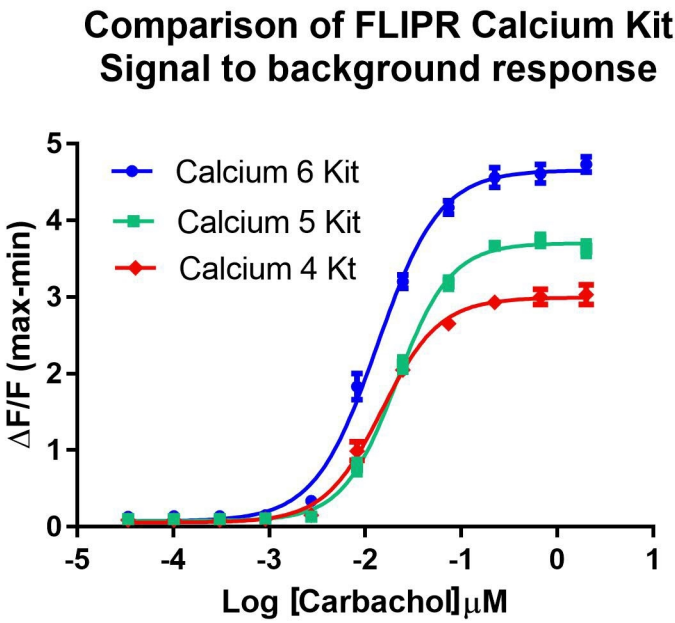


Figure 4-1: Carbachol CRC in CHO M1 cells. Cells were seeded overnight at 25 µL per well in a 384-well black clear bottom-plate. Cells were incubated with 25 µL of each of the three Calcium Assay dyes including probenecid. Calcium 4 and 5 were incubated for 1 hour at 37°C 5% $CO_2$ . Calcium 6 kit was incubated for 2 hours to allow the slightly larger molecule to enter the cell. During simultaneous detection on a FLIPR TETRA system with ICCD camera, a 5X concentration of carbachol was added (12.5 µL /well) to achieve the final indicated concentration $EC_{50}$ values for Carbachol were 13, 15, and 21 µM respectively.

### 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 Tested With Buffer Plus DMSO Show a Signal 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 Evaluation 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 an open reservoir in SBS plate format ‘Boat’ for the presoak.

### Apparent Well-to-Well Variation is Observed

A liquid dispenser compatible with cell handling is recommended for use with all additions off the FLIPR or FlexStation instruments 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.

# Chapter 6: Product Use Limitations and Warranty

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