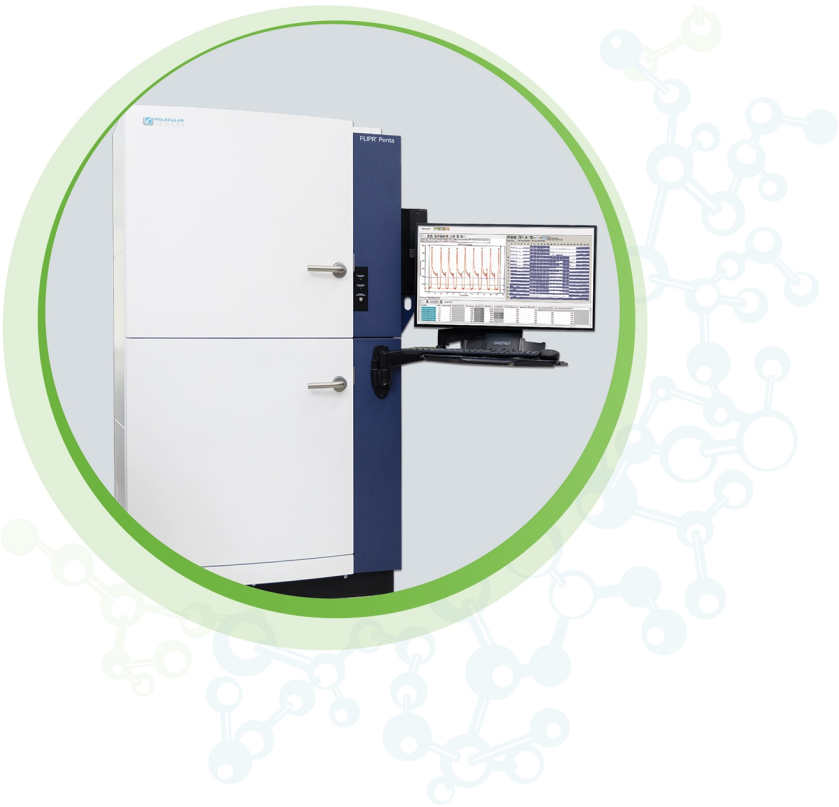

FLIPR Penta

High-Throughput Cellular Screening System

ScreenWorks Software Version 5.1

Protocol Guide

MOLECULAR DEVICES

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September 2020

Product manufactured by Molecular Devices, LLC.
3860 N. First Street, San Jose, California, 95134, United States of America.
Molecular Devices, LLC is ISO 9001 registered.

Contents

Chapter 1: Introduction

The FLIPR® Penta High-Throughput Cellular Screening System provides an automated solution for identifying early leads in the drug discovery process and for evaluating drug efficacy and toxicity. The system supports a variety of assay kits optimized for use on your instrument.

This guide provides detailed information, procedures and optimization guidelines for running the following types of experiments:

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.

You can contact your local representative or Molecular Devices Technical Support at 800-635-5577 (North America only) or +1 408-747-1700. In Europe, call +44 (0) 118 944 8000.

Please have your instrument serial number or Work Order number, and your software version number available when you call.

WARNING! BIOHAZARD. It is your responsibility to decontaminate components of the instrument before you return parts to Molecular Devices for repair. Molecular Devices does not accept items that have not been decontaminated where it is applicable to do so. If parts are returned, they must be enclosed in a sealed plastic bag stating that the contents are safe to handle and are not contaminated.

Chapter 2: FLIPR Calcium 6 Assay Kit Protocol

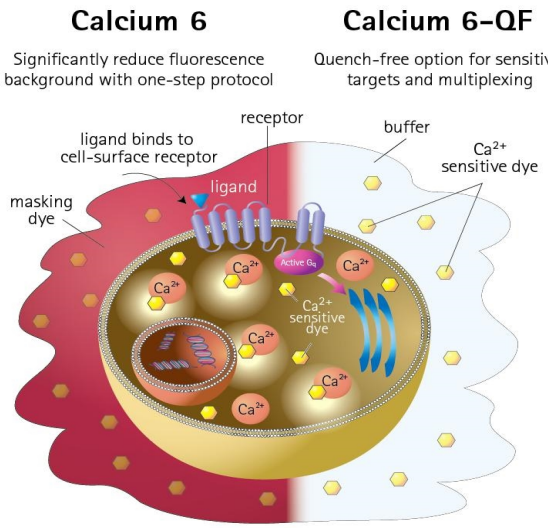
This protocol includes the following information:

About the FLIPR Calcium 6 Assay Kit

Assay Principle

Calcium assays from Molecular Devices use sensitive calcium indicators and masking dyes. The FLIPR Calcium 6 Assay Kit contain a dye formulation that further enhances the calcium flux assay with an increased signal window. Kit components are mixed with buffer and incubated for approximately two hours with cells. During incubation, the indicator passes through the cell membrane, and esterases in the cytoplasm cleave the acetoxymethyl (AM) portion of the molecule. After incubation with the dye, the cells are ready to be assayed. When the target is activated, direct measurement of intracellular fluorescence change due to increased calcium concentration is enabled. The masking dye in the formulation does not enter the cell, but it significantly reduces background originating from residual extracellular fluorescence of calcium indicator, media, and other components. The FLIPR Calcium 6 QF Assay Kit (Quench-Free) formulation is a flexible option for quench-sensitive targets or multiplexing applications.

Some cell lines that have an anion exchange protein require the use of an anion reuptake inhibitor, such as probenecid, to retain commonly used calcium indicators like Fluo-3 and Fluo-4. However, the dye formulation for the FLIPR Calcium 6 Assay Kits is more resistant to such organic anion transporters, and thus little or no probenecid needs to be used for assays run with FLIPR Calcium 6 Assay Kits. This is especially useful for evaluating targets that are sensitive to probenecid, as well as for screening agonists and antagonists.



Increase in cytosolic Ca^{2+} can be detected by FLIPR or FlexStation microplate readers using calcium-sensitive dye indicators

Application

The FLIPR 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. The kit provides a homogeneous assay designed to work with the majority of GPCRs (including Chemokine and other difficult receptors,) sticky compounds and allosteric modulators, as well as with calcium channels. In addition, the new FLIPR Calcium 6 QF Assay Kit formulation is a flexible option for quench-sensitive targets or multiplexing applications.

Table 2-1: FLIPR Calcium 6 Assay Kit Application Choices

Kits	Media containing serum	Assay Buffer	Multiplexing	Probenecid Sensitive Cell Lines
FLIPR Calcium 6 Assay Kit	X	X		X
FLIPR Calcium 6 QF Assay Kit		X	X	X

Materials and Equipment

Kit Components

- FLIPR Calcium 6 Assay Kit
- FLIPR Calcium 6 QF Assay Kit on page 10

FLIPR Calcium 6 Assay Kit

Table 2-2: Components of the FLIPR Calcium 6 Assay Kit

Item	Evaluation Kit (R8194)	Explorer Kit (R8190)	Bulk Kit (R8191)	Express Kit (R8195)
Component A	3 vials	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	1 bottle	—	—

- The entire Evaluation Kit is sufficient for three (3) 96-well, 384-well, or 1536-well microplates. Each vial is sufficient for assaying one (1) 96-well, 384-well, or 1536-well microplate, depending on dead volume of the dispenser used.
- 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, 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, or seventy-five (75) 1536-well microplates, depending on dead volume of the dispenser used.

FLIPR Calcium 6 QF Assay Kit

Table 2-3: Components of the FLIPR Calcium 6 QF Assay Kit

Item	Evaluation Kit (R8194)	Explorer Kit (R8192)	Bulk Kit (R8193)	Express Kit (R8196)
Component A	3 vials	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	1 bottle	—	—
Component C	3 vials	10 vials	10 vials	2 vials

- The entire Evaluation Kit is sufficient for three (3) 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 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, 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, or seventy-five (75) 1536-well microplates, depending on dead volume of the dispenser used.

Materials Required But Not Provided

See Consumables and Accessories on page 79.

Table 2-4: Reagents and Supplies

Item	Suggested Vendor
Dimethyl Sulfoxide (DMSO) is used for dissolving Component A. It is important that the DMSO used is of high quality and stored properly.	Sigma #D8418, or equivalent
Component B*: 20 mM HEPES buffer + 1X Hank's Balanced Salt solution (HBSS), pH 7.4 *Component B is provided for Evaluation kits and 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

Table 2-4: Reagents and Supplies (continued)

Item	Suggested Vendor
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. Tip: With Calcium 6 Kit and Calcium 6-QF Kit, it is possible to run with less probenecid or none at all if the target is sensitive to probenecid. Assay development is required to determine the best concentration.
1536-well low-base black wall, clear bottom assay microplates compatible with the 1536-well pipettor head	Greiner 783092 or equivalent

Storage and Handling

On receipt of the FLIPR Calcium 6 Assay Kit or the FLIPR Calcium 6 QF Assay Kit, depending on your kit contents, store Component A and Component C 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

Experimental Protocol

Quick Start Protocol

To run a FLIPR Calcium 6 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 depending on your kit, do the following:
   - FLIPR Calcium 6 Assay Kit—add an equal volume of Loading Buffer to each well. For example, add 25 µL of Loading Buffer to 25 µL of cells and media for a 384-well microplate.
   - FLIPR Calcium 6 QF Assay Kit—remove the culture media and add 25 µL HBSS + 20mM HEPES followed by 25 µL Dye Loading Buffer.

     Note: Removal of the culture media prevents hydrolysis and increased background in assays run with FLIPR Calcium 6 QF Assay Kit.
4. Return prepared plates to the incubator and incubate two hours at 37°C (98.6°F), 5% CO_2.

5. Prepare compound plates.
6. Run the experiment on a FLIPR Penta instrument, reader.

Cell Handling

The FLIPR Calcium 6 Assay Kit and FLIPR Calcium 6 QF Assay Kit are 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 cells most frequently used 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 2-5 for suggested plating volumes and seeding densities to create an 80-90% confluent cell monolayer before placing the plates in the plate reader.

Table 2-5: 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

FLIPR Calcium 6 Assay Kit

The following procedure is designed for preparation of the FLIPR Calcium 6 Assay Kit Loading Buffer per vial of the Explorer Kit. More volumes for the Bulk Kit and Express Assay Kit are included in Table 2-6.

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 approximately 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.

Table 2-6: Required volumes to formulate FLIPR Calcium 6 Assay Kits

Plate Well-Type	Volumes to Formulate FLIPR Calcium 6 Assay Kits	Evaluation Kit (R8194)	Explorer Kit (R8190)	Bulk Kit (R8191)	Express Kit (R8195)
96 or 384	Volume to dissolve Component A	10 mL	10 mL	10 mL	20 mL
	More required to correct volume*	None	None	90 mL	480 mL
1536	Volume to dissolve Component A	6.5 mL	6.5 mL	10 mL	25 mL
	More required to correct volume*	None	None	55 mL	300 mL
* Molecular Devices recommends rinsing the bottle several times to reduce reagent loss.					

FLIPR Calcium 6 QF Assay Kit

The following procedure is for the preparation of the Explorer Kit for the FLIPR Calcium 6 QF Assay Kit Loading Buffer. Volumes required for the Bulk Kit and Express Assay Kit are included in Table 2-6.

To prepare loading buffer:

1. Remove one vial each of Component A and C from the freezer and equilibrate to room temperature.
2. Equilibrate Component B to room temperature.
3. Dissolve contents of one vial Component A by adding 10 mLs of Component B. You can substitute 1X HBSS Buffer plus 20 mM HEPES for Component B.
4. Dissolve one vial of Component C in 25 µL DMSO and mix by pipetting. Transfer the contents to the same tube as listed in Step 3.
5. Rinse the vial of Component C with 100 µL Component B, and transfer the contents to the same tube as listed in Step 3. Mix by vortexing for approximately 1 to 2 minutes.

Table 2-7: Required volumes to formulate FLIPR Calcium 6 QF Assay Kits

Plate Well-Type	Volumes to Formulate FLIPR Calcium 6 Assay Kits	Evaluation Kit (R8194)	Explorer Kit (R8192)	Bulk Kit (R8193)	Express Kit (R8196)
96 or 384	Volume to dissolve Component A	10 mL	10 mL	10 mL	20 mL
	More required for correct volume	None	None	90 mL	480 mL
	Volume DMSO to dissolve Component C	25 µL	25 µL	250 µL	1.25 mL
1536	Volume to dissolve Component A	6.5 mL	6.5 mL	10 mL	25 mL
	More required for correct volume	None	None	55 mL	300 mL
	Volume DMSO to dissolve Component C	25 µL	25 µL	250 µL	1.25 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 to the appropriate amount of probenecid, 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. See the procedure for making probenecid in Materials Required But Not Provided on page 10. 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.

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.
2. Depending on your kit, do the following:
   - FLIPR Calcium 6 Assay Kit—You do not have to remove the culture media.
   - FLIPR Calcium 6 QF Assay Kit—You must remove the culture media and replace it with HBSS + 20 mM HEPES to maintain the signal.

   Note: Molecular Devices does not recommend washing cells before dye loading. However, 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 2 hours 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 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 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 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 FLIPR Instruments

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

Recommended EMCCD Camera Parameters

Table 2-8: 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

Recommended HS EMCCD Camera Parameters

The following settings are for use in Normal camera mode.

Table 2-9: 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

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

Recommended ICCD Camera Parameters

Table 2-10: Experimental setup parameters for 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

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 on page 86.

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

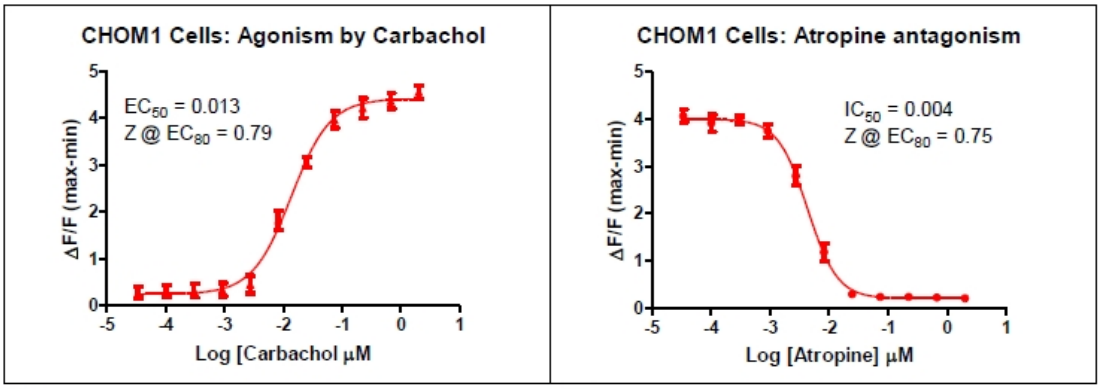
An automated liquid dispenser is recommended for use with additions of cells or dye prior to the assay on the FLIPR Penta System if apparent well-to-well variation is observed. In some cases allowing the plates to stand at room temperature prior to use or adding a single mix cycle in the compound or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

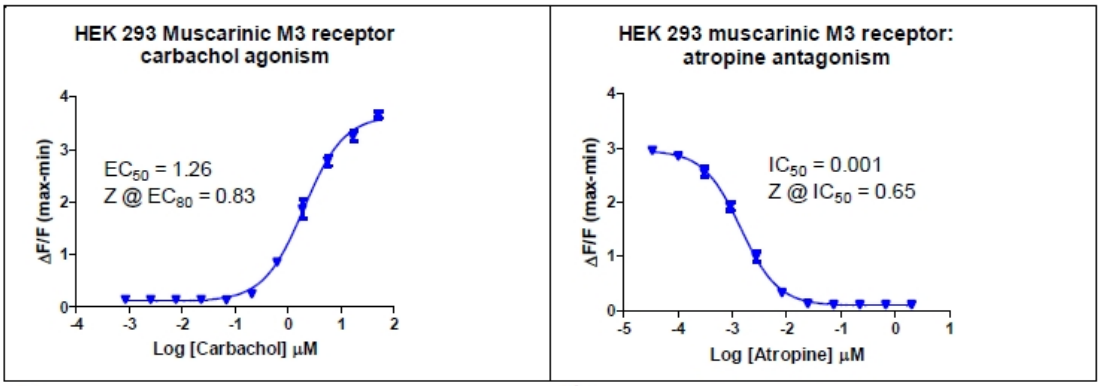
Note: Do not use the same source plate for pre-soaking and compound addition when using a 384 Pipettor head in the FLIPR Penta System. Instead, use a 'Boat' for the pre-soak.

Data Analysis Examples



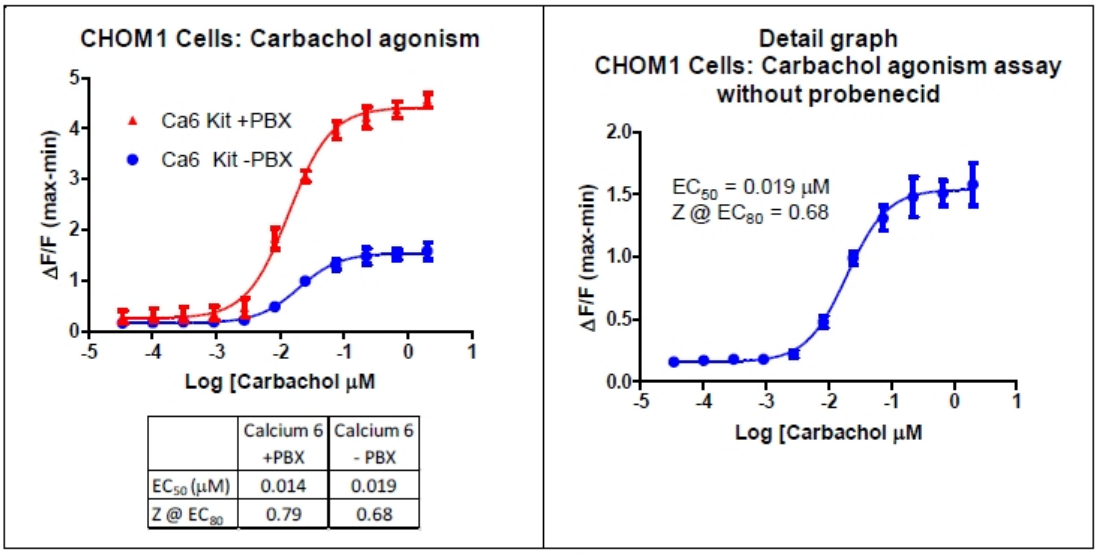
2a 2b

Carbachol concentration response curve in WT3 CHO M1 cells. Cells were seeded overnight at 25 µL per well in a 384-well black-wall clear-bottom plate. On the day of the assay, cells were incubated in media with 25 µL of Calcium 6 Assay Kit loading buffer. All plates were incubated for 2 hours @ 37°C and 5% CO_2. In graph 2a, a volume of 12.5 µL 5X carbachol was added per well as agonist during detection on a FLIPR Tetra instrument with ICCD camera. The Z factor @ EC_{80} was 0.79 and the EC_{50} values were comparable to published values. Graph 2b shows the antagonism response to 50 nM carbachol by atropine. The Z factor @ IC_{50} was 0.75µM. The IC_{50} value is comparable to published values.



3a 3b

HEK-293 cells were seeded overnight at 25 µL per well in a 384-well black wall clear bottom Poly-D Lysine coated plate. On the day of the assay, culture media was removed and the cells were incubated in 25 µL HBSS + 20 mM HEPES and 25 µL of Calcium 6-QF dye. Cells were incubated for 2 hours @ 37°C and 5% CO_2. Graph 3a shows the agonist response of the endogenous muscarinic M3 receptor to carbachol. The EC_{50} value was comparable to other assays. The Z factor at EC_{80} was 0.83 µM. In graph 3b, a 5X volume of the antagonist, atropine, was added per well and the plate was incubated for 10 minutes at room temperature. A 6X concentration of carbachol (0.6 µM final in well) was added as challenge agonist during detection on a FLIPR Tetra instrument with ICCD camera to achieve the final indicated concentration.



	Calcium 6 +PBX	Calcium 6 - PBX
EC_{50} (µM)	0.014	0.019
Z @ EC_{80}	0.79	0.68

4a 4b

Carbachol concentration response curve as seen in graph 2a with additional comparison to an assay run at the same time in Calcium 6 kit dye without probenecid in the loading buffer. In graph 4a, both curves are shown. The EC_{50} values are both within the range of published values and very close to each other. Graph 4b is a larger version of the same curve from the assay run without probenecid. Despite the smaller signal window, the EC_{50} value is conserved and the Z factor @ EC_{80} is 0.68. This suggests that the new dye formulation is less sensitive to the organic-anion transporter and requires less, or even no, anion transporter inhibitors (probenecid) to be present in the assay system. A target that is sensitive to the presence of probenecid in the loading buffer will benefit from the ability to remove it.

Chapter 3: FLIPR Calcium Oscillation Assay in Neurons Protocol

This protocol includes the following information:

About the Calcium Oscillation Assay in Neurons

It has been shown that changes in intracellular calcium levels in neuronal tissue can be used as an important biomarker to investigate the effects of compounds on neuronal network physiology. Calcium oscillations are dependent on an influx of extracellular calcium through L-type voltage-gated calcium channels, and the rising phase of each calcium spike is coincident with a brief burst of action potentials.

In this study, we describe phenotypic assays for neuromodular assessment using human iPSC-derived 3D neural cultures. The 3D culture of human iPSC-derived neural cells is composed of cortical glutamatergic and GABAergic neurons and astrocytes from a single donor source. There has been growing interest in using 3D cell models containing multiple cell types for studying complex biology and tissue architecture due to the ability of cell aggregates to organize and more closely recapitulate key aspects of the human tissue. Importantly, the in vitro model described in this protocol enables detection of consistent spontaneous calcium oscillations and can be used in high-throughput multi-well format for screening of potential neuromodulators.

Fast kinetic fluorescence imaging on the FLIPR Penta System measures the patterns and frequencies of calcium oscillations of neuro–spheroids as monitored by changes in intracellular calcium levels with FLIPR Calcium 6 Dye.

Applications

The FLIPR Calcium 6 Assay Kit uses a newly improved calcium dye formula that further enhances the signal window of the assay and makes difficult assays such as neuronal calcium oscillation more amenable for high throughput screening. The FLIPR Calcium 6 kit provides a homogeneous assay designed to work for the majority of GPCRs, including chemokine and other difficult receptors, sticky compounds and allosteric modulators, as well as with L-type calcium channels and other spontaneous calcium activity.

Required Materials

- Neurons and culture media that pairs with the neurons
- FLIPR Calcium 6 Assay Kit made up at 2x in DPBS (Dulbecco's Phosphate Buffered Saline) for early time points and at 4x in DPBS for the 24-hour time point

  Note: The dye is solubilized in DPBS. Stock compounds are made in DMSO and diluted in culture media.

- FLIPR® Penta High-Throughput Cellular Screening System
- ScreenWorks® Peak Pro™ Software module

Storage and Handling

On receipt of the FLIPR Calcium 6 Assay Kit, store Component A 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 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.

Experimental Protocol

Neuronal Cell Culture Handling

Plate and feed neurons following your developed protocol or the supplier protocol.

In this experiment, 3D neural cultures: StemoniX microBrain® 3D Assay Ready 384-Well plates (Cat# BSARX-AA-0384) were provided by StemoniX, Inc. Plates were shipped pre-plated under ambient conditions. Each well contained a single, uniformly sized human iPSC-derived cortical neural spheroid matured 8-9 weeks.

On the day of arrival, plates were centrifuged for 5 min at 1000 RPM (Sorvall centrifuge), then inspected using a microscope for recovery, decontaminated with 70% ethanol, and unsealed. Afterwards, the media was changed (1/2 of volume, 3 times), and plates were placed into a 37°C 5% CO_2 incubator for 5-7 days.

Media change was performed every second day.

On the day of assay, adjust the volume of cell to 25 µL in a 384-well plate.

Preparing Loading Buffer

The following procedure is designed for preparation of the FLIPR 6 Calcium Assay Kit Loading Buffer per vial of the Explorer Kit. More volumes for the Bulk Kit are included in Table 3-1.

To prepare the loading buffer:

1. Remove one vial of Component A from the freezer and equilibrate to room temperature.
2. Instead of using Component B from the kit, add the FLIPR Calcium 6 Dye to DPBS (Dulbecco's Phosphate Buffered Saline), not provided.
3. Dissolve the contents of one Component A vial by adding 10 mL of DPBS and then mix by vortexing for ~1 minute until the contents of the vial are dissolved.

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

Table 3-1: Required volumes of DPBS to formulate FLIPR 6 Calcium Assay Kits

Plate Well-Type	Volumes to Formulate FLIPR 6 Calcium Assay Kits	Explorer Kit (R8190)	Bulk Kit (R8191)
96 or 384	Volume of DPBS to dissolve Component A* This makes 2x dye to be added in equal volume to the wells with cells	10 mL	100 mL
	Volume of DPBS to dissolve Component A* This makes 4x dye to be added to the wells with cells at 24 hours	5 mL	10 mL
	More DPBS required for correct volume*	None	40 mL
* DPBS not provided in kits.			

Running the Calcium Oscillation Assay in Neurons on a FLIPR Penta Instrument

After incubation, transfer the microplates to the FLIPR Penta 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 Penta instrument with an EMCCD camera, adjust typical average baseline counts to a range from 800 RFU to 1500 RFU.
- 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 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 Penta instrument are listed in the following tables.

Slower addition speeds farther from the cell monolayer or spheroid are recommended to ensure 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. Alternatively, add compounds offline, since the neuron assay is not like a traditional calcium flux assay for GPCR assays where addition at the same time as reading is critical.

Recommended Settings

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

Before starting the experiment, it is important to check the parameters of the oscillating neurons with either a signal test or a short read to ensure you have the proper settings for your experiment, because parameters vary depending on the cells.

Faster addition speeds can lead to better mixing of compounds and lower signal variance across the plate.

Detection Parameters	ICCD Camera		Standard, EMCCD Camera		HS, EMCCD Camera	
	96-well	384-well	96-well	384-well	96-well	384-well
Exposure (sec)	0.05	0.05	0.2	0.2	0.4	0.4
Camera Gain	50	50	80-130	80-130	1.5-4	1.5-4
Camera Gate	22	22	—	—	—	—
Read Time Interval	0.2	0.2	0.3	0.3	1.0	1.0
Number of Reads	3000	3000	2000	2000	3000	3000
Excitation LED (nm)	470-495	470-495	470-495	470-495	470-495	470-495

Detection Parameters	ICCD Camera		Standard, EMCCD Camera		HS, EMCCD Camera	
	96-well	384-well	96-well	384-well	96-well	384-well
Emission Filter (nm)	515-575	515-575	515-575	515-575	515-575	515-575
LED Intensity (%)	50	50	80	80	80	80
Camera Mode	—	—	—	—	Normal High Speed	Normal High Speed
Stabilize Time (sec)	—	—	—	—	50	50

When using the **HS EMCCD camera** in HighSpeed camera mode, since biology varies, run a Protocol Signal Test with a sample plate to determine your specific protocol exposure settings. Use the recommended settings as a starting point. If your experiment could have brightness that exceeds your signal test sample plate, then reduce the exposure settings accordingly so that your brightest signal does not exceed 43,000 counts. Refer to the *FLIPR Penta High-Throughput Cellular Screening System User Guide* topic "Understanding HS EMCCD Camera Protocol Saturation."

Addition Parameters	96-Well Plate	384-Well Plate
Compound Concentration (Fold)	5X	5X
Addition Height (µL)	210-230	35-45
Tip Up Speed (mm/s)	10	10
Compound Addition Volume (µL)	50	12.5
Dispense Speed (µLs) Adherent Cells	50–100	30–40
Dispense Speed (µL/s) Non-adherent Cells	10–20	10–20

Calcium Flux Assay in Neurons

Calcium oscillation in neurons was assessed using FLIPR Calcium 6 Kit (Molecular Devices). Kinetics of Ca^{2+}-flux were determined at 515-575 nm following excitation at 470-495 nm for 10 minutes at a frequency of 10 Hz using the FLIPR Penta High-Throughput Cellular Screening System with the high-speed camera. The exposure time per read was 0.2 seconds, the gain was set to 40.50, and the excitation intensity was set to 80%. The deck temperature was kept at a constant 37°C. Early effects on calcium oscillations were measured at 60 minutes following initial exposure to compounds. The dye was made up in DPBS. For the early time points, cells were pre-loaded with FLIPR Calcium 6 Dye and added at 2x concentration to an equal amount of media on the cells for 2 hours before compound addition. Baseline for calcium oscillations without compounds was typically measured before compound addition. For 24-hour experiments, cells were exposed to compounds at appropriate concentrations for 22 hours before the addition of Calcium 6 Dye. Calcium 6 Dye (4x concentration) was added for an additional 2 hours with extra volume of compounds to keep the compound concentration the same.

Compounds

To keep the concentration of compounds consistent, treatments were performed at one hour and at 24 hours, using replacement of half-volumes of media with 2x final concentrations of compounds. Final concentration of DMSO in the wells was 0.15%.

Quantitative Data Evaluation

Representative descriptors such as peak count (per 10 minutes), average peak amplitude, average peak width (at 10% amplitude), average peak spacing (time between peaks), average peak rise time (from 10% to 90% amplitude), and average peak decay time (from 90% to 10% amplitude) were derived using the ScreenWorks Peak Pro Software Software module.

Results

The neuronal cells in the StemoniX microBrain 3D spheroids generate spontaneous synchronized calcium oscillations. We used kinetic fluorescence imaging on the FLIPR Penta System to measure the patterns and frequencies of the Ca^{2+} oscillations of neuro-spheroids as monitored by changes in intracellular Ca^{2+} levels with FLIPR Calcium 6 dye after a two-hour incubation period. A set of known neuromodulators was tested, including agonists and antagonists of NMDA, GABA, and kainate receptors.

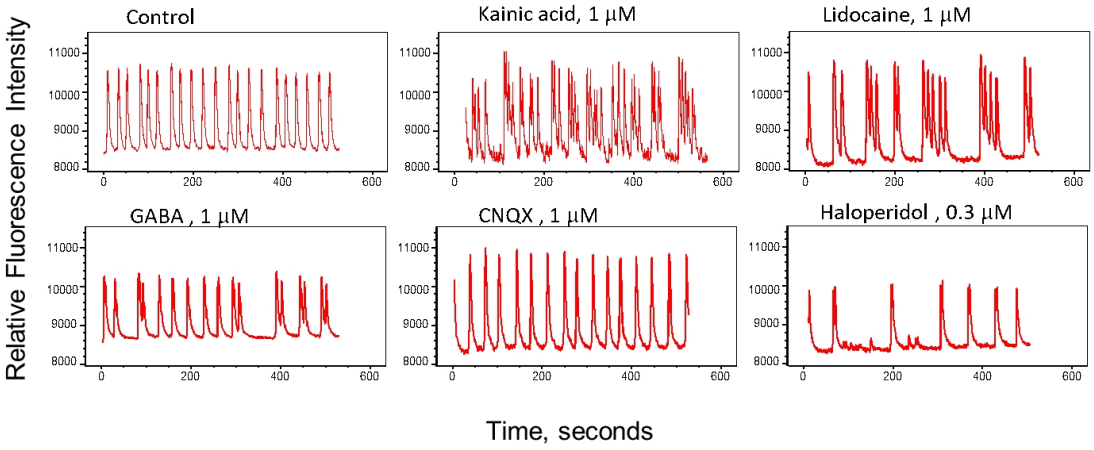


Figure 3-1: Calcium oscillation frequency by the spheroids was measured over a 10-minute period after a 1-hour incubation with compound. Oscillation patterns from a subset of six compounds.

Notice the differences in the oscillation patterns when compared to the control. IC_{50} values and the mechanism of action of each compound, as shown in Table 3-2.

Table 3-2: Neuroactive Compounds

Compound	Mechanism of Action	Effect on Calcium Oscillation (IC_{50}, µM)
Control	—	NA
Kainic Acid	Kainate receptor agonist	2.66
Lidocaine	Na+ channel blocker	9.47
GABA	GABA agonist and endogenous inhibitory neurotransmitter	5.93
CNQX	Kainate receptor antagonist	2.05
Haloperidol	D2 Antagonist and NMDA sub antagonist	0.13

Chapter 4: EarlyTox™ Cardiotoxicity Kit Protocol

This protocol includes the following information:

About the EarlyTox Cardiotoxicity Kit

There is a need for cardiotoxicity and cardio-discovery assays that use more biologically relevant cell-based models to help with the development of new chemical entities and ensure drug safety. The EarlyTox Cardiotoxicity Kit has been optimized to provide a robust, high throughput method for measuring the impact of pharmacological compounds on stem cell derived cardiomyocytes, including measurement of the peak rate, amplitude, peak width, and other critical parameters.

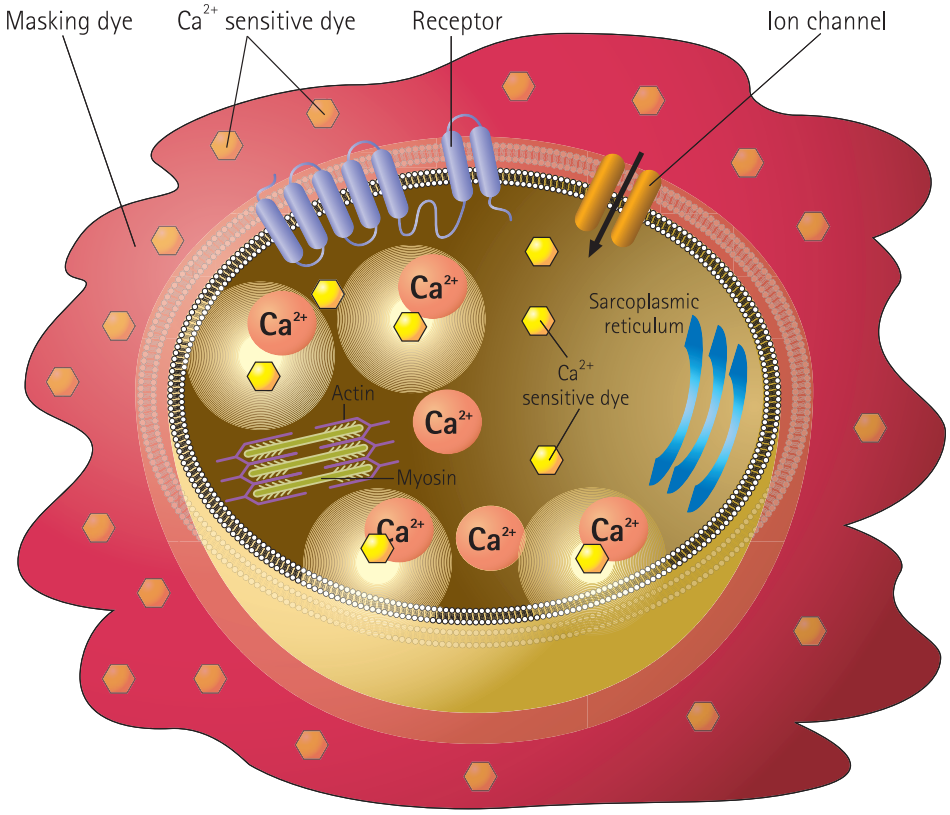
Calcium assays from Molecular Devices employ sensitive calcium indicators and masking dyes. The EarlyTox Cardiotoxicity Kit contains an optimized dye formulation that increases the signal window while having minimal non-specific toxicity and impact on the peak rate and other peak characterization parameters. Other commercially available dyes and kits demonstrate non-specific effects on multiple parameters of cellular beating, all within the time-frame required for measurement of compound effects. The cardiotoxicity dye is mixed with the provided buffer and incubated for approximately two hours with cells, before assay. During incubation, the indicator passes through the cell membrane, and esterases in the cytoplasm cleave the AM portion of the molecule. The masking dye does not enter the cell, but significantly reduces background originating from residual extracellular fluorescence of the calcium indicator, media, or other components. This removes the requirement for washing the cells in order to obtain a robust signal window. As cardiomyocyte peak rates are sensitive to buffer changes; it is critical for the cells to remain in media to produce consistent high quality results.

After incubation with the dye, the cells are ready to be assayed. Compounds are added and measurements taken using the FLIPR® Penta High-Throughput Cellular Screening System. Direct measurement of intracellular fluorescence change due to changes in intracellular calcium concentrations is enabled. This change in calcium concentration correlates to the oscillation rate and oscillation pattern of the cardiomyocyte, as further discussed in Assay Principle on page 31.

Three reference compounds are included in the kit:

- Isoproterenol, a non-selective ß-adrenergic agonist that induces positive chronotropic and inotropic effects. Isoproterenol (isoprenalin) is used for the treatment of bradycardia (slow heart rate) or heart block.
- Propranolol, a non-selective blocker of both β1-adrenergic and β2-adrenergic receptors. It inhibits the action of agonists of β-adrenergic receptors.
- Sotalol is a drug used for treatment of arrhythmias. It is a non-selective competitive β-adrenergic receptor blocker that also inhibits potassium channels. It changes the normal contraction pattern.

Assay Principle



EarlyTox Cardiotoxicity dye binds with calcium ions as they enter the cell cytoplasm enabling measurement of the change in calcium concentration. During a single calcium oscillation event, Ca^{2+} is released into the cytoplasm after the sarcoplasmic reticulum is stimulated. Calcium binds with troponin activating the sarcomere, and the cell contracts. Synchronously, there is an increase in fluorescent signal as dye binds to free calcium in the cytosol. Cellular relaxation occurs on removal of calcium from the cytosol by calcium uptake pumps of the sarcoplasmic reticulum and by calcium exchange with extracellular fluid. The fluorescent signal decreases as calcium concentration decreases. As the cycle repeats, more fluorescent peaks synchronous with calcium oscillation are observed.

Note: This is a simplified description of the cellular actions occurring. For a more detailed explanation of the methods of calcium changes and their relation to relaxation-contraction we recommend more in-depth reading of the literature related to the actions of cardiac muscle physiology. Some readings are referenced in Bibliography—Selected Readings on page 40.

Advantages

Molecular Devices developed the EarlyTox Cardiotoxicity Kit to deliver a robust, high-throughput, biologically relevant assay solution enabling earlier prediction of compound toxicity and efficacy.

- Eliminate cardiotoxic compounds and identify potential drug candidate
- Study functional profiles in a biorelevant cardiotoxicity assay
- High-throughput: measure 384 samples in minutes rather than hours
- Minimal non-specific effect of dye on calcium oscillation characteristics
- Reduced well-to-well variation, improved data quality
- Enhanced signal dynamic range
- Minimal cell perturbation (no wash required)
- Adaptable for use 96-well, 384-well, and 1536-well formats

Applications

The EarlyTox Cardiotoxicity Kit assay is designed to detect the effects of compounds on the beat characteristics of cardiomyocytes. It employs a calcium sensitive dye to monitor changes in intracellular calcium concentrations, which in turn are synchronous with cell beating. Assay results can help with the detection of efficacy or toxicity of compounds. The EarlyTox Cardiotoxicity Kit uses a newly improved calcium dye formula and patented quench technology that further enhance the signal window, minimize impact on beating rate over time, and make difficult assays more amenable for high-throughput screening.

This EarlyTox Cardiotoxicity Kit has been tried with the following types or sources of cardiomyocytes:

- iCell® Cardiomyocytes and iCell® Cardiomyocytes^2, human induced pluripotent stem (iPSC) cell-derived cardiomyocytes from Fujifilm Cellular Dynamics International.
- Cardiomyoctyes derived from H7 human embryonic stem cells (hESC).
- Cor.4U® Cariomyocytes from Ncardia, Ag.

Materials and Equipment

Kit Components

Table 4-1: Components of the EarlyTox Cardiotoxicity Kits

Item	Explorer Kit (R8210)	Bulk Kit (R8211)
Component A Loading dye	2 vials	2 vials
Component B Dilution buffer: 1X DPBS (with Ca^{2+} and Mg^{2+}) plus 20 mM HEPES buffer, pH 7.4	1 bottle	1 bottle
Reference compounds: isoproterenol, sotalol, propranolol. When dissolved in 50 µL DPBS or DMSO, each aliquot will result in 20 mM concentration.	3 vials	3 vials

- The entire Explorer Kit (R8210) is sufficient for two 96-well or 384-well plates. Each vial is sufficient for one 96-well or 384-well plate.
- The entire Bulk Kit (R8211) is sufficient for ten 96-well, 384-well, or 1536-well plates. Each vial is sufficient for assaying five 96-well, 384-well, or 1536-well plates.

Storage and Handling

On receipt of the EarlyTox Cardiotoxicity Kit, store the contents at –20°C (-4°F). Under these conditions the reagents are stable for six (6) months in the original packaging.

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

Experimental Protocol

Quick Start Protocol

To run a cardiac calcium oscillation assay protocol:

1. Plate the thawed iPSC derived cardiomyocyte cells in 96-well or 384-well microplates as recommended by the cell provider protocol and incubate for 10 to 14 days at 37°C, 5% CO_2 or as directed by your protocol or a manufacturer's protocol. Feed the cells every second day or as recommended in the cell provider protocol.
2. Prepare the loading buffer on the same day as the assay.
3. Remove the cell plates from the incubator.

- (Optional) Change the media in the microplates before the experiment. Add 25 µL of new media.
- EarlyTox Cardiotoxicity Kit: Add an equal volume of loading buffer to each well, for example: 25 µL of loading buffer to 25 µL of cells and media for a 384-well plate.

4. Return the plates to the incubator and incubate two hours at 37°C, 5% CO_2.

5. For assay development, prepare the compound plates, include reference concentration response curves of reference compounds as positive controls. Molecular Devices recommends half log serial dilutions starting at: isoproterenol 10 µM, sotalol 100 µM, and propranolol 100 µM.
6. Run the experiment on a FLIPR Penta System, or other compatible Molecular Devices instrument. See Compatible Molecular Devices Instruments.

Cell Handling

The EarlyTox Cardiotoxicity Kit assay is designed to work with stem cell derived cardiomyocytes or primary cardiomyocytes. Standard procedures vary across laboratories and Molecular Devices recognizes that a variety of cell handling conditions might be used at the discretion of the user. This section contains general guidelines for preparing cells for use with the assay kit. iPSC-derived cardiomyocytes or ES-derived cardiomyocytes are the most frequently used cells. They are generally plated 10 to 14 days before an experiment and then incubated in a 5% CO_2, 37°C incubator. See specific cell vendor protocols for recommended cell plating and handling. Molecular Devices recommends optimizing the assay and cell conditions for specific applications.

Table 4-2: General Plating Volumes and Seeding Densities

Cell Type (cells/well)	96-well microplate (100µL growth medium)	384-well microplate (25µL growth medium)
iCell Cardiomyocytes (human-iPS cell derived, from Cellular Dynamics International)	40000 to 80000	8000 to 15000
H7 cardiomyocytes (human-ESC cell derived)	Not determined at time of publication	40000 to 60000

Preparing the Loading Buffer

The following procedure is designed for the preparation of the EarlyTox Cardiotoxicity Kit loading buffer per vial of the Explorer Kit. More volumes for the Bulk Kit are included in Table 4-3.

To prepare the loading buffer:

1. Remove one vial of EarlyTox Cardiotoxicity Dye (Component A) from the freezer and equilibrate to 37°C. Also equilibrate Component B to 37°C.
2. Dissolve contents of the loading dye (Component A) vial by adding 10 mL of dilution buffer (Component B) or 1X DPBS Buffer plus 20 mM HEPES. Mix by vortexing ~1 to 2 minutes until contents of vial are dissolved.

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

Table 4-3: Required Volumes of DPBS plus 20 mM HEPES (or Component B)

Microplate Format	DPBS + 20mM HEPES or Component B	Explorer Kit (R8210)	Bulk Kit (R8211)
96-well or 384-well	Volume to dissolve loading dye (Component A)	10 mL	10 mL
	More required for correct volume	None	40 mL

3. Dissolve contents of the reference compounds by adding 50 µL of DMSO or 1X DPBS Buffer plus 20 mM HEPES. Mix by pipetting until contents of vial are dissolved. Final concentrations in the vial is 20 mM of each compound.

Note: As an alternative, 10 mM of compound solutions can be prepared by adding 100 µL DMSO or DPBS.

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

Loading Cells Using Loading Buffer

To load cells using loading buffer:

1. Remove cell plates from the incubator. It is not necessary to remove the culture media. Add an equal volume of loading buffer to each well: 100 µL per well for 96-well microplates, 25 µL for 384-well microplates.
2. After adding dye, incubate cell plates for ~ 2 hours at 37 °C (loading time can be optimized for specific cell source and experiment).

CAUTION! Do not wash the cells after dye loading.

Note: Applicable compound dilutions can be prepared during this step.

3. (Optional) Read the plate after two hours of loading dye (pre-read before compound addition) as suggested below.
4. Add 5x concentrations of compounds manually or using fluidics. Incubate 10 to 30 minutes (optimal time for compound treatment can be optimized for specific cell source and experiment).
5. Read the microplate.
6. (Optional) Repeat the plate read as many times as needed with a read only protocol to monitor compound effects at different time points.

Note: Keep the plate in the incubator between reads.

Diluting Reference Compounds and Preparing the CRC Plate

To dilute reference compounds and prepare the CRC plate:

1. Dissolve reference compounds in 50 µL of DMSO or Component B buffer to make 20 mM stock concentration.
2. Dilute stock concentrations to prepare 5x solutions so that when added to the cells you have the following final concentrations:
   Molecular Devices recommends performing half log serial dilutions and to start each concentration response curve (CRC) at the following highest dose:
   - Isoproterenol at 10 µM
   - Sotalol at 100 µM
   - Propranolol at 100 µM

Running the Cardiac Beating Assay on a FLIPR Penta Instrument

To run a cardiac beating assay:

1. Pre-warm the instrument deck to 37°C before the experiment.
2. After the incubation, transfer the microplates directly to the FLIPR instrument and start the assay as described in the instrument user guide.
3. When running a signal test before an experiment, adjust typical average baseline counts depending on the camera installed in your system.
   - For a FLIPR Penta instrument with an EMCCD camera, adjust typical average baseline counts to a range from 800 to 1100 RFU.
   - For a FLIPR Penta instrument with an ICCD camera, adjust typical average baseline counts to a range from 5000 to 7000 RFU.
   - For a FLIPR Penta instrument with an HS EMCCD camera, adjust the typical average baseline to about 5,000 RFU.
4. Run a couple of read only assays to see how the amplitude shows with the baseline you have set, and adjust as needed.
5. Molecular Devices recommends the experiment setup parameters for each FLIPR instrument as listed in Table 4-4. However, further assay development and adjustment of the volume, height, and speed of dispense are recommended to optimize the individual cell response.

Recommended Settings for the FLIPR Instrument

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

Table 4-4: Experiment setup parameters for the FLIPR Penta Instrument

Parameter	96-well microplate	384-well microplate
Camera Mode	**HS EMCCD camera:** HighSpeed	**HS EMCCD camera:** HighSpeed
Excitation LED (nm)	470–495	470–495
Emission Filter (nm)	515–575	515–575
Camera Gain	EMCCD camera: 130 HS EMCCD camera: 4 ICCD camera: Fixed at 2000	EMCCD camera: 130 HS EMCCD camera: 4 to 50 ICCD camera: Fixed at 2000
Exposure (sec)	0.05 **Note:** 0.05 second exposure or less depending upon the acquisition rate (interval) chosen. 30 Hz or 0.03 seconds with a 0.02 second exposure interval is an optimal read for peak detail of iPSC Cardiomyocytes.	0.05 or less (see note)

Table 4-4: Experiment setup parameters for the FLIPR Penta Instrument (continued)

Parameter	96-well microplate	384-well microplate
LED Intensity (%)	EMCCD camera: 80 HS EMCCD camera: 50 ICCD camera: 50	EMCCD camera: 80 HS EMCCD camera: 50 ICCD camera: 50
Camera Gate (%) (ICCD camera only, adjust so camera does not saturate)	22	22
Addition Volume (µL)	50	12.5
Compound Concentration (Fold)	5X	5X
Addition Height (µL)	210 to 230	35 to 45
Addition Speed (µL/sec)	Adherent Cells: 50	Adherent Cells: 30
Tip-Up Speed (mm/sec)	10	10

When using the HS EMCCD camera in HighSpeed camera mode, since biology varies, run a Protocol Signal Test with a sample plate to determine your specific protocol exposure settings. Use the recommended settings as a starting point. If your experiment could have brightness that exceeds your signal test sample plate, then reduce the exposure settings accordingly so that your brightest signal does not exceed 43,000 counts. Refer to the *FLIPR Penta High-Throughput Cellular Screening System User Guide* topic "Understanding HS EMCCD Camera Protocol Saturation."

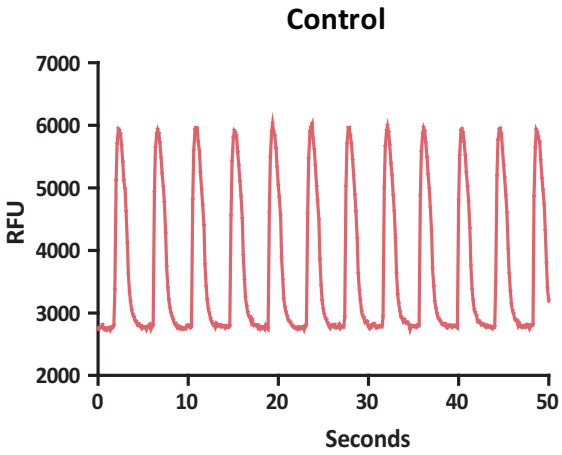
Data Analysis Examples

iPSC derived cardiomyocyte assay experimental protocol

The data below were generated using iCell® Cardiomyocytes, human induced pluripotent stem (iPS) cell-derived cardiomyocytes from Cellular Dynamics International. The cells were thawed and plated in a volume of 25 µL in a 384-well, black-wall, clear-bottom microplate based on vendor protocol. Cells were incubated and fed for 10 days before the assay. On the day of the assay, dye loading buffer was prepared and all assay preparation steps are as described in Experimental Protocol, see page 33.

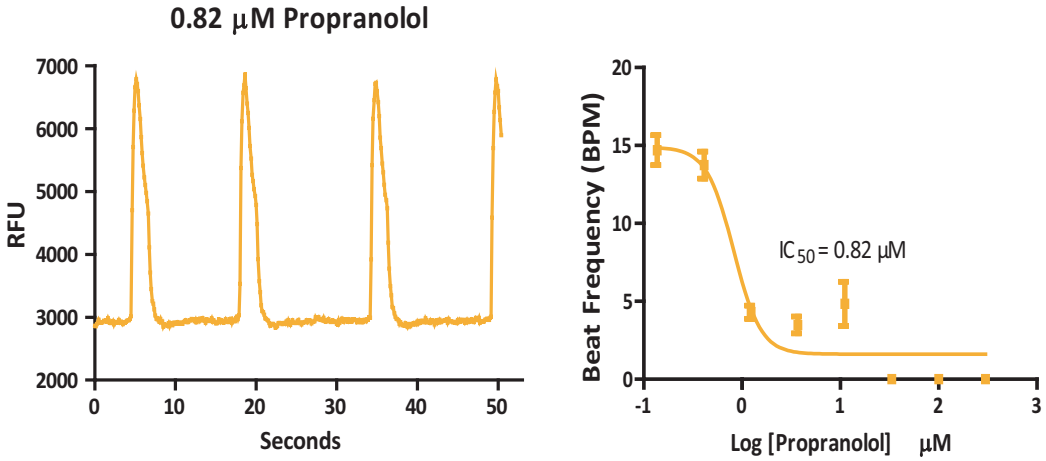
For the FLIPR Penta data and FLIPR Tetra data that follows, cell plates were transferred to the instrument, and compounds were added simultaneous with detection. Cells were returned to the incubator for 20 minutes, and then read again following a protocol for 800 reads at 0.05 second intervals without pipetting. ScreenWorks Software was used to analyze and export either beats per minute (BPM) or Time Sequence data to GraphPad Prism for graphing. EC_{50} or IC_{50} values were determined for control and each of the three reference compounds. Beat profiles were also analyzed as they can enable identification of the effects of compounds that interfere with potassium or other ion channels such as hERG blockers.

Untreated Control iPSC Cardiomyocytes 20 Minutes Post Compound Addition



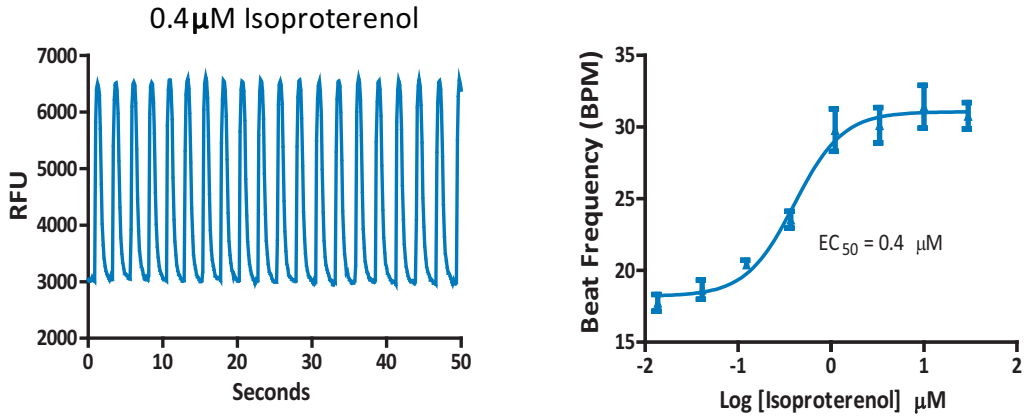
In the figure above, changes in fluorescent signal reflect the contractions or beating of the entire well of iPSC derived cardiomyocytes loaded with Cardiotoxicity Dye. As the cells contract and relax, changes in intracellular calcium concentration are reflected by the change in the fluorescent signal. EarlyTox Cardiotoxicity Kit can be used to record signal traces using the FLIPR Tetra instrument or FLIPR Penta instrument for several hours after compound addition as it has minimal non-specific toxicity compared to other available dyes. In addition, the larger signal window provided by the dye can help with determination of cell behavior when the signal is small.

iPSC Cardiomyocytes Treated with Propranolol Exhibit a Slower Peak Frequency



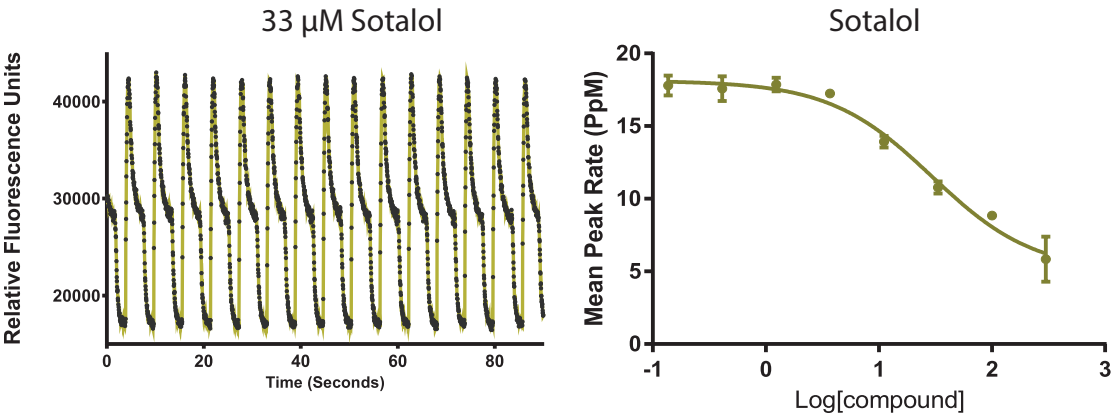
In the figure above, cells are treated with propranolol, which inhibits the action of ß-adrenergic agonists. It slows the beat frequency or contraction rate of the cells. IC_{50} values are determined from the change in contraction rate (beat frequency) vs. concentration of compound.

iPSC Derived Cardiomyocytes Treated with Isoproterenol Show Increased Peak Frequency



In the figure above, isoproterenol is shown to increase the contraction rate of the cells. Isoproterenol is a positive chronotrope that is a non-selective ß-adrenergic agonist. This data point was measured 20 minutes after compound addition, however BPM can be re-measured over several hours to determine delayed toxicity effects. EC_{50} values are determined from the peak frequency vs. concentration of compound.

iPSC Cardiomyocytes Treated with Sotalol Show Decreased Peak Frequency and Changed Beat Pattern



In the figure above, cells were treated with sotalol, a non-selective ß blocker that also inhibits potassium channels and changes the normal beat pattern. The IC_{50} value was calculated in ScreenWorks Peak Pro Software Version 2.0. EarlyTox Dye enables the identification of compounds that might have an unintended effect of affecting potassium or other ion channels early in the drug discovery process. The above experiment was run with the HS EMCCD camera at 30 Hz or an interval of .03 seconds to capture peak detail.

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Chapter 5: FLIPR Membrane Potential Assay Kit Protocol

This protocol includes the following information:

About FLIPR Membrane Potential Assay Kit

The FLIPR® Membrane Potential Assay Kit from Molecular Devices provides a fast, simple and reliable fluorescence-based secondary assay for detecting changes in voltage across the cell membrane. These kits are designed to work in association with many receptors and ion channels as well as both adherent and non-adherent cell lines. The assay is a mix-and-read procedure in which cells are incubated with the kit reagents for 30 minutes after which the signal is detected using a FLIPR® High-Throughput Cellular Screening System .

There are no intermediate wash steps involved and, typically, the assay is complete one minute after addition of the agonist.

Conventional protocols for evaluating changes in membrane potential are technique-sensitive, multi-step procedures consisting of preparing large batches of dye and introducing dye into the compound plate. Several problems are routinely encountered with the conventional methods, all of which add to experimental variability.

Because ion channel activity is highly sensitive and potentially impacted by subtle chemical changes, two FLIPR® Membrane Potential Assay Kits, Red and Blue, are available to select the optimal conditions for your delicate ion channel targets. Both formulations use Molecular Devices different proprietary quench technology to enhance signal windows and yield acceptable Z-scores to screen a variety of targets, including TRP, ligand-, cyclic nucleotide- and voltage-gated channels. Both formulations, however use the same proprietary indicator dye. Evaluate both assay kits to decide which formulation is best for your target.

Problems in a conventional assay protocol include:

- Slow response time of traditional dyes used
- Extensive pre-read soaking procedure
- Requirement for precise temperature control
- Variation in fluorescence according to ionic concentrations

Molecular Devices has developed the FLIPR Membrane Potential Assay Kits to maximize cell line/channel/compound applicability while eliminating causes of variability in the data and reducing the number of steps in the conventional protocol.

Advantages of the FLIPR Membrane Potential Assay Kits include:

- More reproducible data because washing is not required
- Faster dye response time
- No cumbersome pre-assay preparation
- Ease of use at room temperature to physiological temperatures
- Fewer steps in the assay resulting in higher sample throughput

Required Materials

The following materials are required for running a membrane potential assay:

Item	Source
FLIPR® Penta System with Membrane Potential Optics Kit installed: FLIPR® Penta System LED Module 510–545 nm FLIPR® Penta System Emission Filter 565–625 nm	Molecular Devices See Consumables and Accessories on page 79
One of the following Membrane Potential Assay Kits: Evaluation Kit MP Blue, explorer MP Blue, bulk MP Red, explorer MP Red, bulk	Molecular Devices R8128 R8042 R8034 R8126 R8123
Clear, flat-bottom, black- or clear-wall 1536-, 384- or 96-well plates	See Consumables and Accessories on page 79
Clear polypropylene source plates	See Consumables and Accessories on page 79
FLIPR® Penta System pipette tips, 96, 384 or 1536 gasket	See Consumables and Accessories on page 79
Cells in suspension	—
Test compounds	Specific to receptor
Growth medium	Major Laboratory Supplier (MLS)
Incubator	MLS
Centrifuge	MLS
Pipettor and sterile tips suitable for use with microplates	MLS
1 N NaOH	MLS

Note: The Kit includes masking dye technology covered by issued and pending patents including US 6,420,183, US 7,063,952 and EP 0,906,572, licensed exclusively from Bayer AG to Molecular Devices.

Table 5-1: Components of the FLIPR Penta Membrane Potential Assay Kit

Item	Explorer Kit (MP Blue R8042) (MP Red R8126)	Bulk Kit (MP Blue R80342) (MP Red R8123)
Component A	10 vials	10 vials
Assay Buffer (Component B) 20 mM HEPES buffer + 1X Hank's Balanced Salt solution (HBSS), pH 7.4	1 bottle	—

FLIPR Membrane Potential Assay Cell Preparation

Cell Densities

A variety of cell handling conditions may be adopted at your discretion based on standard operating procedures in the laboratory. Non-adherent cells are typically plated on the day of experiment on a coated plate (for example, poly-d-lysine or collagen) to assist cell adherence to the plate bottom. We recommend you then centrifuge the plates at 1000 rpm for up to 4 min with the brake off. Adherent cells are seeded the day prior to an experiment and incubated in a 5% CO_2, 37°C incubator overnight. To create an 80%–90% confluent cell monolayer, it is recommended the cells be seeded at densities shown in the table below.

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

FLIPR Membrane Potential Assay Kit Dye Loading

Preparing Loading Buffer

The following procedure is designed for preparation of the Loading Buffer for either of the FLIPR Membrane Potential Assay Kits, BLUE (R8034) or RED (R8123) in the Bulk format.

Blue or Red

To prepare loading buffer for one Bulk Kit vial:

1. Prepare 100 mL Hanks Balanced Salt Solution (HBSS) + 20 mM HEPES. Adjust to pH 7.4 (Component B) with 1 N NaOH.

   Note: Depending upon the cell type and application, the Assay Buffer as provided with the FLIPR Membrane Potential Assay Kit might not be the ideal choice. If so, alternative buffers, such as Tyrode's or PBS, can be used at your discretion to achieve optimal results.

2. Dissolve completely the contents of each Component A vial by adding 100 mL of 1x (96-wells and 384-wells) or 67 mL of 3x (1536-wells) Assay Buffer. Mix by repeated pipetting until the contents are completely dissolved.

   Note: For best results, dissolve the contents of each vial with 10 mL of 1x Assay Buffer and vortex for at least 30 seconds. Wash the vial several times using 1x Assay Buffer to yield a total volume of 100 mL (96-wells or 384-wells) or 67 mL (1536-wells).

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

   CAUTION! 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.

Table 5-2: Required volumes to formulate FLIPR Membrane Potential Assay Kits

Plate Well-Type	Volumes to Formulate FLIPR Membrane Potential Assay Kits	Evaluation Kit (R8128)	Explorer Kit (Red R8126) (Blue R8042)	Bulk Kit (Red R8123) (Blue R8034)
96 or 384	Volume to dissolve Component A (1 vial)	10 mL	10 mL	10 mL
	More required for correct volume	None	None	90 mL
1536	Volume to dissolve Component A	6.5 mL	6.5 mL	10 mL
	More required for correct volume	None	None	55 mL

Loading the Cells Using Loading Buffer

To load the cells:

1. Remove the cell plates from the incubator or centrifuge. Do not remove the supernatant. Add Loading Buffer to each well (100 µL per well for 96-well plates, or 25 µL for 384-well or 2 µL for 1536-well plates).

   Note: Although Molecular Devices does not recommend washing cells before dye loading, growth medium and serum factors can be washed away before adding the Loading Buffer, provided residual volumes after the wash step are as described. Alternatively, cells can be grown in serum-free conditions.

2. Incubate the cell plates for 30 minutes at 37°C (98.6°F).

   Note: In some cases, incubation at room temperature may improve results.

CAUTION! Do not wash the cells after dye loading.

Running the Membrane Potential Assay on FLIPR Instruments

Before incubation, ensure that the FLIPR instrument is equipped with the 510–545 nm LED module and 565–625 nm emission filter. See LED module and emission filter installation instructions in your instrument user guide, or a picture diagram can be sent to you by contacting Molecular Devices Technical Support.

To run a membrane potential assay protocol:

1. Choose the 510–545/565–625 excitation/emission wavelengths respectively from the **Settings** process in ScreenWorks® Software. After incubation, transfer the plates directly to a FLIPR instrument and start the Membrane Potential assay.
2. Place the filter holder in its correct position in the FLIPR instrument.
3. Do the assay signal test before the experiment. The LED intensity, exposure time, and the gate or gain can be adjusted to get the needed RFU range.
   - For a FLIPR Tetra or FLIPR Penta instrument with an EMCCD camera, adjust the typical average baseline counts to a range from 1500 RFU to 2500 RFU.
   - For a FLIPR Penta instrument with an HS EMCCD camera, adjust the typical average baseline counts to a range from 2,500 to 5,000 RFU.
   - For a FLIPR Tetra or FLIPR Penta instrument with an ICCD camera, adjust the typical average baseline counts to a range from 8000 RFU to 10000 RFU.
4. Update the assay signal test to transfer the adjusted settings to the protocol.

5. Before reading the microplate, set up your FLIPR instrument using the recommended experimental setup parameters listed in Recommended Settings for FLIPR Instruments on page 46.

Recommended Settings for FLIPR Instruments

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

Note: The addition speeds are faster than in the conventional protocol because of the increased robustness of the cells after the new loading procedure. Faster addition speeds can lead to better mixing of compounds and lower signal variance across the plate.

Detection Parameters	EMCCD Camera	HS EMCCD Camera*	ICCD Camera
Read Mode	Fluorescence	Fluorescence	Fluorescence
Camera Mode	—	Normal	—
Excitation Wavelength (nm)	510–545	510–545	510–545
Emission Wavelength (nm)	565–625	565–625	565–625
Excitation Intensity (%)	50[1]	40[1]	50[1]
Camera Gain	50	4	2000
Gate Open	—	—	6[1]%
Exposure Length (s)	0.4[1]	0.02	0.53[1]
Read Interval (s)	1	1	1
* HS EMCCD camera is only compatible with the FLIPR Penta System. Contact Molecular Devices for upgrade information. 1. Can be adjusted to increase or decrease the signal if low RFUs or saturation problems occur.			

Addition Parameters	96-Well Plate	384-Well Plate	1536-Well Plate
Compound Addition Volume (µL)	50	12.5	1
Compound Concentration (Fold)	5x	5x	7x
Dispense Speed (µL/s) Adherent Cells	50–100	20–40	2–8
Dispense Speed (µL/s) Nonadherent Cells	10–50	5-20	1-4
Dispense Height	180	30	1

Troubleshooting the FLIPR Membrane Potential Assay Kit

This section presents solutions to problems that users may encounter when running membrane potential 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 may result from dislodging cells from the wells during addition. Shortening incubation times, plating cells on poly-D-lysine plates or slowing the dispense speed should solve the problem in this case.

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 plate, 25 µL for 384 well plate, or 2 µL for 1536-well plates.

CAUTION! Increasing the final in-well concentration of the Loading Buffer may decrease the response of the assay. If only one addition is required, then add the appropriate volume of buffer before addition one.

Fluorescence Increase

An increase of fluorescence may be observed upon buffer only challenge. This increase is most likely due to a response of endogenous ion channels to increasing ionic strength. Patch clamping data supports this observed change. The choice of cells and expression levels of endogenous channels can greatly influence resting and changing membrane potentials. Match the compound addition buffer to the buffer in the cell plate (culture medium plus dye loading buffer) so there is no change in ion concentration upon compound addition.

No Response

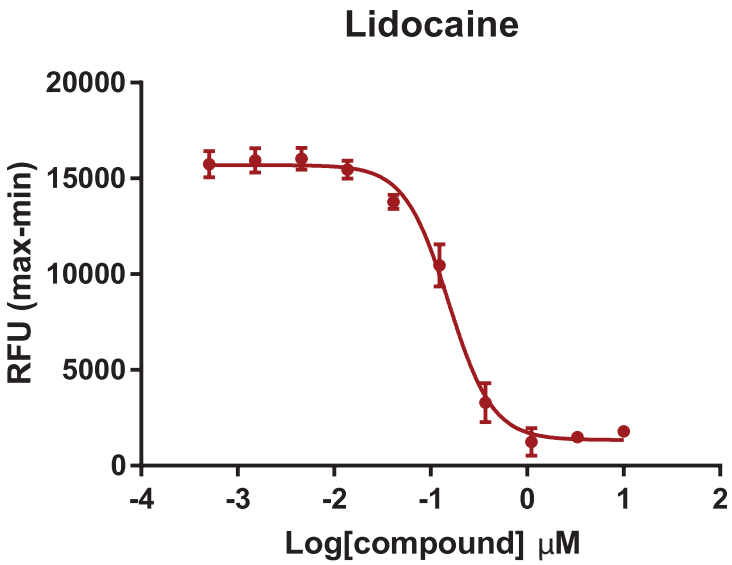
Not all assays work well with all cell lines or channels. To address this problem, we have developed two different assays to maximize the opportunity for a successful assay. If you have not tried both FLIPR Membrane Potential Assay Kit formulations, Blue and Red, then first try the alternate kit to determine if your cell line is compatible. The FLIPR Membrane Potential Evaluation Assay Kit (R8128) contains both formulations in an Explorer Kit format to facilitate testing of cell lines, channels, and compounds. If you do not see a change in fluorescence with either formulation, then we suggest changing some of the assay conditions.

Recommendations include: replacing media before dye loading, longer incubation and assay times; preparing compounds in the loading buffer; and choosing different buffers such as Tyrode's or specific ion-free buffers. For example, if studying a calcium channel, dye load cells using a calcium-free buffer and prepare your compound plate using a calcium containing buffer.

Effect of DMSO on Membrane Potential Assays

High concentrations of DMSO are toxic to many cell lines and affect compound mixing. Also, DMSO can induce physiological changes in cells (for example, differentiation). If the test compound preparation contains DMSO, it is important to titrate the DMSO to determine the maximum concentration that can be used with each indicator cell line and test compound. With some cell lines that have been tested, there was no effect on signal level up to 1% DMSO final concentration.

Data Analysis Examples



Modulation of NaV1.5 channel in CHL cells by lidocaine. Veratridine can be used instead of voltage to open sodium channels as part of a high throughput membrane potential screening strategy to identify modulating compounds. Compared to the IC_{50} value in an electrophysiology assay, the IC_{50} value of lidocaine block in the FLIPR Membrane Potential Assay is 154 nM and is much smaller, which is consistent with previous publications and suggests most NaV1.5 channels in CHL are not at a completely closed state.

Chapter 6: FLIPR Potassium Assays Kit Protocol

This protocol includes the following information:

About the FLIPR Potassium Assay Kit

Advantages

Currently, thallium-based potassium assay methodologies are slow and laborious, and can require multiple replacements of assay buffers or medium. In addition, the assay quality is often challenged by well-to-well variations and a small signal window. Therefore, a fast, easy-to-use, and sensitive cell-based assay specific to potassium channels is highly desirable for high-throughput screening. The FLIPR Potassium Assay Kit provides a true homogeneous assay using a no-wash format, with a number of key features including:

- Direct, functional measurement of potassium channel activity
- Homogeneous, no-wash protocol
- Enhanced signal window and higher signal-to-noise ratio
- Rapid procedure with less hands-on time
- Reduced well to well variation
- Ease of use with both adherent and non-adherent cells
- Adaptable for use in 96-well, 384-well, or 1536-well formats

Applications

The FLIPR Potassium Assay Kit provides a homogeneous assay for measuring potassium channel activities in a no-wash format. It is designed to work for the majority of ligand-gated or voltage-gated potassium channels.

Assay Principle

The assay exploits the permeation of thallium ions (Tl^+) through both voltage-gated and ligand-gated potassium (K^+) channels. In this assay, a novel, highly-sensitive Tl^+ indicator dye is used. This dye produces a bright fluorescent signal upon the binding to Tl^+ conducted through potassium channels. The intensity of the Tl^+ signal is proportional to the number of potassium channels in the open state. Therefore, it provides a functional indication of the potassium channel activities. In addition, one of the Molecular Devices proprietary masking dyes is employed to further reduce background fluorescence for improved signal-to-noise ratio.

During the initial dye-loading step, the acetoxymethyl (AM) ester tagged Tl^+ indicator dye enters the cells through passive diffusion. Cytoplasm esterases cleave the AM ester and relieve its active thallium-sensitive form. To activate the potassium channels, the cells are then stimulated with either a mixture of K^+ and Tl^+, or a ligand in the presence of Tl^+. The increase of fluorescence in the assay represents the influx of Tl^+ into the cell specifically through the potassium channel, providing a functional measurement of potassium channel activity.

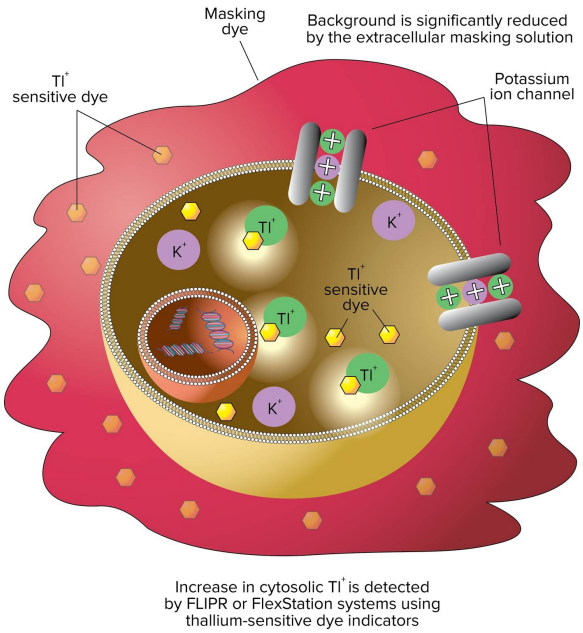


Figure 6-1: Assay principle of the FLIPR Potassium Assay Kit

Materials and Equipment

Kit Components

Table 6-1: Components of the FLIPR Potassium Assay Kits

Item	Evaluation Kit (R8330)	Explorer Kit (R8222)	Bulk Kit (R8223)
Component A	2 vials	10 vials	10 vials
Component C	2 vials	10 vials	10 vials
Chloride-free Assay Buffer (5X)	1 bottle	1 bottle	1 bottle
Potassium Sulfate solution 200 mM of K_2SO_4 in water	1 bottle	1 bottle	1 bottle
Thallium Sulfate solution 50 mM of Tl_2SO_4 in water	1 bottle	1 bottle	1 bottle
Assay Buffer (Component B) 20 mM HEPES buffer + 1X Hank's Balanced Salt solution (HBSS), pH 7.4	1 bottle	1 bottle	None

- The entire Evaluation Kit is sufficient for two (2) 96-well or 384-well microplates. The entire Explorer Kit is sufficient for ten (10) 96-well or 384-well microplates. Each vial is sufficient for one (1) microplate.
- The entire Bulk Kit is sufficient for one-hundred (100) 96-well or 384-well microplates. Each vial is sufficient for ten (10) microplates.

Materials Required But Not Provided

See Consumables and Accessories on page 79.

Table 6-2: Reagents and Supplies

Item	Suggested Vendor
Dimethyl Sulfoxide (DMSO) is used for dissolving Component A. It is important that the DMSO used is of high quality and stored properly.	Sigma #D8418, or equivalent
Component B*: 20 mM HEPES buffer + 1X Hank's Balanced Salt solution (HBSS), pH 7.4 *Component B is provided for Evaluation kits and 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 HH 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.

Storage and Handling

On receipt of the FLIPR Potassium Assay Kit, store Component A and Component C at -20°C (-4°F), and store the 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

DANGER! Thallium sulfate is harmful if swallowed or touched. Disposal of this product, solutions, and any by-products must comply with the requirements of environmental-protection and waste-disposal legislation, and any regional local authority requirements. Exercise care when handling this product 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

Experimental Protocol

Quick Start Protocol

To run a potassium assay protocol:

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

Cell Handling

The Molecular Devices FLIPR Potassium Assay Kit is designed to work with many cell types, both adherent and non-adherent. Standard procedures vary across laboratories, and Molecular Devices recognizes that a variety of cell handling conditions can be used at the discretion of the user. This section provides general guidelines for preparing cells for use with the FLIPR Potassium Assay Kit.

Adherent cells are the most frequently used cells with the kits. They are generally plated the day before an experiment and then incubated in a 37°C, 5% CO_2 incubator overnight. See Table 6-3 for suggested plating volumes and seeding densities to create an 80% to 90% confluent cell monolayer for the assay in a FLIPR® Penta instrument.

Table 6-3: Suggested Plating Volumes and Seeding Densities

Cell Type (cells/well)	96-well microplate (100 µL growth medium)	384-well microplate (25 µL growth medium)
Adherent cells	20000 to 80000	5000 to 20000
Non-adherent cells	40000 to 200000	10000 to 50000

For non-adherent cells, Molecular Devices recommends centrifuging cells from culture medium and re-suspending the pellet in the culture medium or applicable buffer of choice on the day of the experiment. Cells can be dye-loaded in a tube or while plated. Molecular Devices recommends that after the cells are plated, centrifuge the microplates at 100 x g for up to 4 minutes, with the brake off. As an alternative, 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 (for example, poly-D-lysine or collagen) to make sure that they are well attached to the microplate bottom.

Preparing the Loading Buffer

To prepare the loading buffer:

1. Remove one vial each of Component A and Component C from the freezer, and then equilibrate to room temperature.
2. Equilibrate Component B to room temperature.
   For the Bulk Kit, prepare 100 mL of 20 mM HEPES plus 1X HBSS, pH 7.4 as Component B.

3. Dissolve the contents of the Component C vial in DMSO, and then mix thoroughly by vortexing.
   - For the Evaluation Kit or Explorer Kit, use 30 µL of DMSO.
   - For the Bulk Kit, use 300 µL of DMSO.
4. Combine the vial of Component A with 10 mL of the Component B buffer.
   For the Bulk Kit, use only 10 mL of the prepared Component B buffer in this step.
5. Combine the Component C solution from step 3 to the Component A solution from step 4, and then mix by vortexing for ~1 to 2 minutes until the contents of the vial are dissolved.
   It is important that the contents are completely dissolved to ensure reproducibility between experiments.
6. For Bulk kit only, combine the solution from step 5 with the remaining 90 mL of the prepared Component B buffer, and then mix thoroughly.

Note: If the cells require probenecid (such as CHO or other cells containing an organic anion transporter), then add probenecid in the Loading Buffer to 5 mM so that the final in-well working concentration 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 53.

Note: 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.

Loading Cells Using Loading Buffer

To load cells using loading buffer:

1. Remove the cell plates from the incubator or centrifuge.
2. Add an equal volume of Loading Buffer to each well: 100 µL per well for 96-well plates and 25 µL per well for 384-well plates.

   Note: Molecular Devices does not recommend washing cells before dye loading. However, 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 20mM HEPES buffer + 1X HBSS, pH 7.4 before adding the Loading Buffer.

3. After adding dye, incubate the cell plates for 1 hour at room temperature. The loading time should be optimized for each cell line and target.

   Note: Do NOT wash the cells after dye loading.

Preparing the Thallium Sulfate Source Plate

To introduce Tl^+ into the assay plate, prepare a 5X solution by diluting ligands or K_2SO_4 (for voltage-gated targets only) with Tl_2SO_4 in 1X Chloride-free Buffer.

Concentrations of Tl_2SO_4 and K_2SO_4 (for voltage-gated targets only) used for assays should be optimized for each target channel. Molecular Devices suggests a titration of Tl^+ or a combination of Tl^+ and K^+ at 0.5 mM to 3 mM Tl^+ and 5 mM to 30 mM K^+ (final concentration in wells). See Optimization Guidelines on page 59.

Note: Since thallium sulfate and potassium sulfate have two equivalents of cation per mole, consider them as 2X in their respective cation concentrations.

DANGER! Thallium sulfate is harmful if swallowed or touched. Disposal of this product, solutions, and any by-products must comply with the requirements of environmental-protection and waste-disposal legislation, and any regional local authority requirements. Exercise care when handling this product 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

Running the FLIPR Potassium Assay

After incubation, transfer the microplates to the FLIPR Penta instrument and start the FLIPR Potassium Assay.

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

- For a FLIPR Penta instrument with an EMCCD camera, adjust the typical average baseline counts to a range from 1500 RFU to 2500 RFU.
- For a FLIPR Penta instrument with an HS EMCCD camera, adjust the typical average baseline counts to a range from 2,500 to 5,000 RFU.
- For a FLIPR Penta instrument with an ICCD camera, adjust the typical average baseline counts to a range from 8000 RFU to 10000 RFU.

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

Before reading the microplate, set up the FLIPR instrument using the recommended experimental setup parameters listed in Table 6-4.

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 Penta system using the following recommended experiment settings. Your settings depend on the camera installed in your instrument.

Table 6-4: Experimental setup parameters for the FLIPR Penta instrument

Parameter	96-well microplate	384-well microplate
Excitation LED (nm)	470–495	470–495
Emission Filter (nm)	515–575	515–575
Camera Mode	EMCCD camera: NA HS EMCCD camera: Normal ICCD camera: NA	EMCCD camera :NA HS EMCCD camera: Normal ICCD camera: NA
Camera Gain	EMCCD camera: 70 to 150 HS EMCCD camera: 4 ICCD camera: Fixed at 2000	EMCCD camera: 70 to 150 HS EMCCD camera: 4 ICCD camera: Fixed at 2000
Exposure (sec)	EMCCD camera: 0.1 to 0.5 HS EMCCD camera: 0.2 ICCD camera: 0.4	EMCCD camera: 0.1 to 0.5 HS EMCCD camera: 0.2 ICCD camera: 0.04
LED Intensity (%)	EMCCD camera: 50 to 100 HS EMCCD camera: 40 ICCD camera: 40	EMCCD camera: 50 to 100 HS EMCCD camera: 40 ICCD camera: 40
Camera Gate (%) **(ICCD camera only)**	6	6
Addition Volume (µL)	50	12.5
Compound Concentration (Fold)	6x	6x
Addition Height (µL)	120 to 160	30 to 40
Addition Speed (µL/sec)	Adherent Cells: 60 to 100 Non-Adherent Cells: 20 to 40	Adherent Cells: 30 to 40 Non-Adherent Cells: 10 to 20
Tip-Up Speed (mm/sec)	20	20

Data Analysis Example

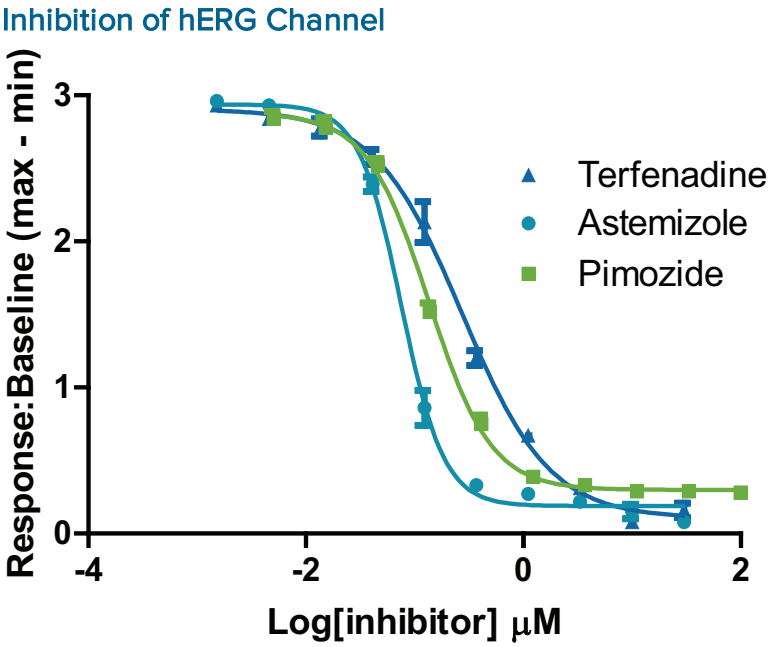


Figure 6-2: hERG Channel Blockers

Compound	IC_{50} (nM) FLIPR Instrument	IC_{50} (nM) Electrophysiology
Terfenadine	274	332
Astemizole	76	62
Pimozide	135	147

In this experiment, the concentration-dependent inhibition of hERG channels by three reference compounds was analyzed with the FLIPR Potassium Assay Kit. The hERG channel was stably expressed in the Chinese hamster ovary (CHO) cells, and all compounds were incubated with cells for 30 minutes after 1 hour of dye loading at room temperature. Stimulus buffer containing 1X chloride-free buffer and final concentrations of 10 mM K^+, and 1 mM Tl^+ was added to the wells while reading on the FLIPR Tetra Instrument. The IC_{50} values derived from this assay were compared to the electrophysiology results collected with the IonWorks Barracuda Instrument.

Optimization Guidelines

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

Data Analysis

Correct data analysis is crucial to correctly assess the functionality of the assay. It is very important to choose the correct time point to calculate final data. In the assay, the assay background trace (optimized Thallium only or maximal inhibition condition) shows a slow upward slope over the course of the assay. Using a late time point results in a reduced assay window impacting the quality of the data and also the IC_{50} determination.

Molecular Devices recommends using a time point that results in the greatest assay window as defined as Max signal vs. Thallium only trace. Identification of the optimal time point can be done by assessing the raw data manually or by using the Negative Control Correction feature in the FLIPR® ScreenWorks® Software. This feature subtracts the assigned background curve, resulting in bell shaped traces. It can be used for identification of the optimal time point or as a general analysis tool. Using Negative Control Correction, the Max-Min analysis automatically uses the data at the optimal time point.

If other analysis methods are preferred, the optimal time point determined can be manually chosen (maximum of bell shaped curve).

Use of the Negative Control Correction feature to determine the optimal time point and signal window for the assay

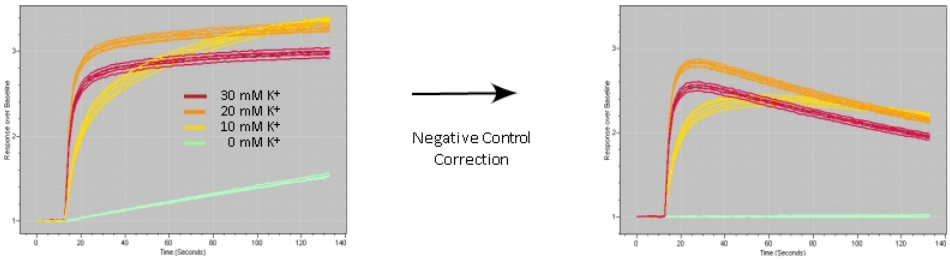


Figure 6-3: Before and after Negative Control Correction

$K_V1.3$ channel activity was measured with 2 mM Tl^+ and different K^+ concentrations. The data are displayed with and without Negative Control Correction.

Determination of Potassium and Thallium Concentrations

Potassium channels are grouped into several classes that have very different properties. It is very important to determine the target-specific optimal concentrations of Tl^+, or Tl^+ and K^+ for each assay. To determine the optimal concentrations to use for the assay, Molecular Devices recommends setting up an initial experiment with a matrix of ligand and Tl^+ or K^+ and Tl^+ at 5 mM to 30 mM K^+ and 0.5 mM to 3M mM Tl^+ (final concentration in the wells).

Titration of K^+ and Tl^+ concentrations to optimize assay performance for different targets

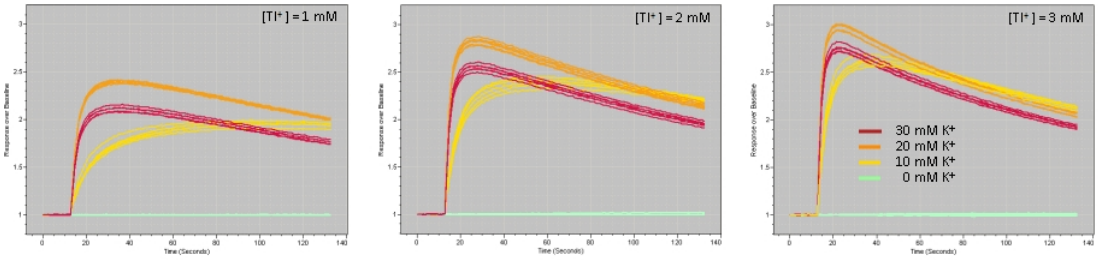


Figure 6-4: K_V1.3 Channel Activity Assay

K_V1.3 channel activity was measured with a titration of different Tl^+ and K^+ concentrations. The data are displayed with Negative Control Correction.

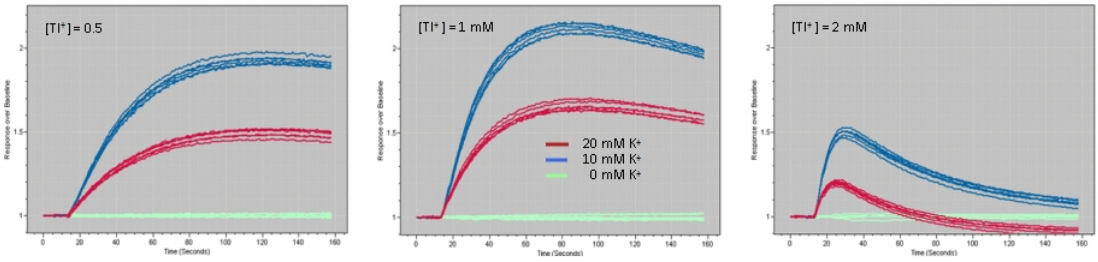


Figure 6-5: hERG Channel Activity Assay

hERG channel activity was measured with a titration of different Tl^+ and K^+ concentrations. The data are displayed with Negative Control Correction.

Chapter 7: FURA-2 QBT™ Calcium Kit Protocol

This protocol includes the following information:

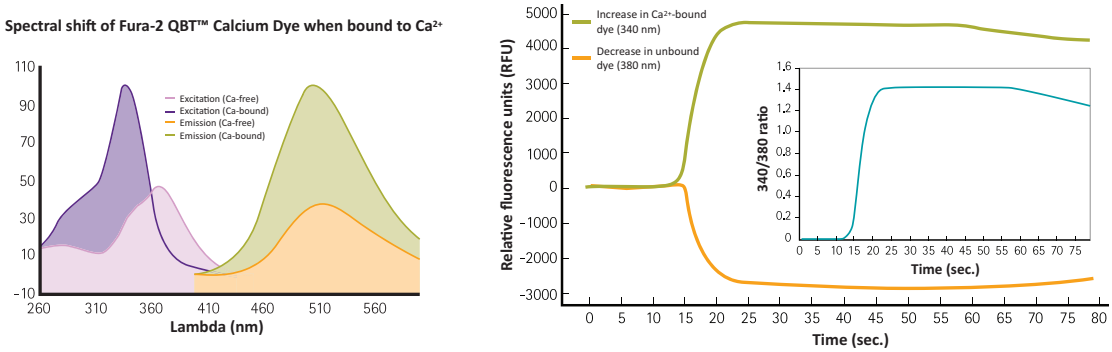
About the Fura-2 QBT Calcium Kit

Molecular Devices calcium assays employ sensitive calcium indicators and a proprietary masking dye. The Calcium Kits contain Fura-2, acetoxymethyl (AM), a cell permeable calcium indicator that is ratiometric, and UV-light excitable. The Ca^{2+}-bound Fura-2 dye is excited at 340 nm, and the unbound form at 380 nm. The emitted light is measured at around 510 nm. With increasing Ca^{2+} concentration in the cytoplasm upon Ca^{2+}-flux there is a spectral shift in the dye and the fluorescence intensity at 340 nm excitation/510 nm emission increases while the fluorescence intensity at 380/510 nm decreases. By using the ratio of the fluorescent intensities produced by the two excitation wavelengths, assay variability from factors such as uneven dye loading and cell number variations are reduced.

The masking dye does not enter the cell, but significantly reduces background, originating from residual extracellular fluorescence of calcium indicator, media, and other components. This removes the requirement for washing of cells before dye loading and before detection found with standard Fura-2 assays. The homogenous assay format delivers a simplified assay protocol, higher throughput, data results with a larger signal window, and reduced well-to-well variation.

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. After incubation with the dye, the cells are ready to be assayed. When the target is activated, direct measurement of intracellular fluorescence change due to increase in concentration of intracellular calcium bound to dye and decrease in concentration of calcium not bound to dye is enabled.

Assay Principle



Fura-2 QBT™ dye is excited at 340 nm and 380 nm, with emission at 510 nm. Upon calcium flux, changes in intracellular calcium concentration can be measured. Ca^{2+}-bound Fura-2 is excited at 340/510 nm, and the unbound form at 380/510 nm. Upon calcium-flux, the Ca^{2+} concentration in the cytoplasm increases, and the fluorescence intensity at 340/510 nm increases, as there is more Ca^{2+} available for the dye to bind with. The 380/510 nm intensity decreases as the concentration of the unbound form of the dye decreases. By using the ratio of fluorescence intensities produced by excitation at two wavelengths, factors such as uneven dye distribution and cell number variation are minimized because they can have an effect on both measurements to the same extent.

Advantages

Conventional Fura-2 calcium mobilization protocols are multi-step procedures that start by pre-washing the cells, loading them with Fura-2 AM, followed by extensive cell washing before running the assay. This type of wash protocol can induce the following problems:

- Cells removed from microplates 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 the Fura-2 QBT Calcium Kit to eliminate the cause of data variability and reduce the number of steps in the conventional wash protocol using Fura-2. Building on the experience of masking technology, the Fura-2 QBT Calcium Kit uses the proven quench technology, as used in the FLIPR Assay Kits, in combination with the Fura-2, AM indicator. This homogenous approach introduces the following improvements over competitive kits as well as conventional wash protocol assays:

- 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 G-protien coupled receptor (GPCR) targets and calcium channels
- Adaptable for use in 96-well and 384-well formats

Applications

The Fura-2 QBT Calcium Kit provides a homogeneous ratiometric assay for measurement of changes in intracellular calcium concentration. It is designed to work with GPCRs and calcium channels.

Materials and Equipment

Kit Components

Table 7-1: Components of the Calcium Kits

Item	Explorer Kit (R8197)	Bulk Kit (R8198)
Component A	10 vials	10 vials
Component B 1X Hank's Balanced Salt solution (HBSS) plus 20 mM HEPES buffer, pH 7.4	1 bottle	None

- The entire Explorer Kit (R8197) is sufficient for ten (10) 96-well or 384-well microplates. Each vial is sufficient for one microplate.

- The entire Bulk Kit (R8198) is sufficient for one-hundred (100) 96-well or 384-well microplates. Each vial is sufficient for ten microplates.

Materials Required But Not Provided

Table 7-2: Reagents and Supplies

Item	Suggested Vendor
Component B*: HBSS Buffer (1X Hank's Balanced Salt solution plus 20 mM HEPES buffer) pH 7.4 *Component B is provided for Explorer kits only.	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) might be required with 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.

Storage and Handling

On receipt of the Fura-2 QBT Calcium Kit, store the Component A contents at -20°C (-4°F) and store Component B contents at 4°C (39.2°F). Under these conditions, the reagents are stable for six (6) 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 (without probenecid) for up to 5 days 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

Experimental Protocol

Quick Start Protocol

To run a Fura-2 protocol:

1. Plate cells in microplates and incubate overnight at 37°C, 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, 25 µL of Loading Buffer to 25 µL of cells and media for a 384-well microplate.
4. Return prepared plates to the incubator and incubate one hour at 37°C, 5% CO_2.
5. Prepare compound plates.
6. Run the experiment on a FLIPR® Penta instrument.

Cell Handling

The Fura-2 QBT Calcium Kit is designed to work with many cell types, both adherent and non-adherent. Standard procedures vary across laboratories and recognizes that a variety of cell handling conditions can be used at the discretion of the user. This section, provides general guidelines for preparing cells for use with the Fura-2 QBT Calcium Kit.

Adherent cells are the most frequently used cells with the kits. They are generally plated the day before an experiment and then incubated in a 37°C, 5% CO_2 incubator overnight. See Table 7-3 for suggested plating volumes and seeding densities to create an 80% to 90% confluent cell monolayer before placing the plates in a FLIPR® Penta instrument.

Table 7-3: 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, Molecular Devices recommends centrifuging cells from culture medium and re-suspending the pellet in the culture medium or applicable buffer of choice on the day of the experiment. Cells can be dye-loaded in a tube or while plated. Molecular Devices recommends that after the cells are plated, centrifuge the microplates at 100 x g for up to 4 minutes, with the brake off. As an alternative, 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, for example poly-D-lysine or collagen, to make sure that they are well attached to the microplate bottom.

Preparing the Loading Buffer

The following procedure is designed for preparation of Loading Buffer per vial of the Explorer Kit. More volumes for the Bulk Kit are included in Table 7-4.

To prepare the Explorer Kit 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.

Table 7-4: Required Volumes of HBSS plus 20 mM HEPES (or Component B) for a 96-well or 384-well microplate

Volumes to Formulate Fura-2 Kit	Explorer Kit (R8197)	Bulk Kit (R8198)
Volume to dissolve Component A	10 mL	10 mL
More required for correct volume	None	90 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 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 64. 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:

1. Remove cell plates from the incubator or centrifuge.
2. Add an equal volume of Loading Buffer to each well: 100 µL per well for 96-well plates and 25 µL per well for 384-well plates.

   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 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 Fura-2 Assay on a FLIPR Penta Instrument

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

Do the assay signal test before the experiment. The two LED sets (Read Mode 1 and Read Mode 2) need to be adjusted independently. 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 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 and exposure setting the same.
- For a FLIPR Penta instrument with an HS EMCCD camera, adjust typical average baseline counts to a range from 3,000 RFU to 5,000 RFU for each LED set. Keep the gain setting and exposure setting the same.
- For a FLIPR Penta instrument with an ICCD camera, adjust baseline counts to a range from 6000 RFU to 9000 RFU. % Gate Open can be adjusted.

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

Suggested experimental setup parameters for each FLIPR Penta 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

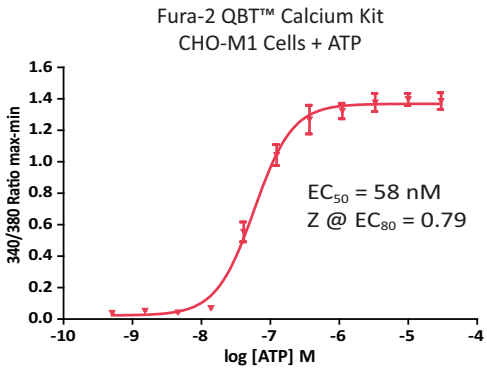
Before reading the microplate, set up your FLIPRPenta using the following recommended experiment settings. Your settings depend on the camera installed in your instrument.

Table 7-5: Experimental setup parameters for a 96-well or 384-well microplate on a Penta instrument

Parameter	EMCCD Camera	HS EMCCD Camera	ICCD Camera
Camera Mode	NA	Normal	NA
Exposure (sec) for the 335–345 LED	0.2 to 0.4	0.2	0.2 to 0.4
Exposure (sec) for the 380–390 LED	0.08 to 0.2	0.2	0.08 to 0.2
Camera Gain	120 to 180	1.5	Fixed at 2000
Camera Gate (%) for the 335–345 LED	NA	NA	70 to 90
Camera Gate (%) for the 380–390 LED	NA	NA	5 to 20
Addition Volume (µL) for 96-well microplate	50	50	50
Addition Height (µL) for 96-well microplate	210 to 230	210 to 230	210 to 230
Addition Speed (µL/sec) Adherent Cells for 96-well microplate	50 to 100	50 to 100	50 to 100
Addition Volume (µL) for 384-well microplate	12.5	12.5	12.5
Addition Height (µL) for 384-well microplate	35 to 45	35 to 45	35 to 45
Addition Speed (µL/sec) Adherent Cells for 384-well microplate	30 to 40	30 to 40	30 to 40
Compound Concentration (Fold)	5x	5x	5x
Excitation LED (nm)	335–345 380–390	335–345 380–390	335–345 380–390
Emission Filter (nm)	475–535	475–535	475–535
LED Intensity (%) for the 335–345 LED	70 to 100	100	70 to 100
LED Intensity (%) for the 380–390 LED	40 to 60	20	40 to 60
Tip-Up Speed (mm/sec)	20	20	20
Addition Speed (µL/sec) Non-Adherent Cells	10 to 20	10 to 20	10 to 20

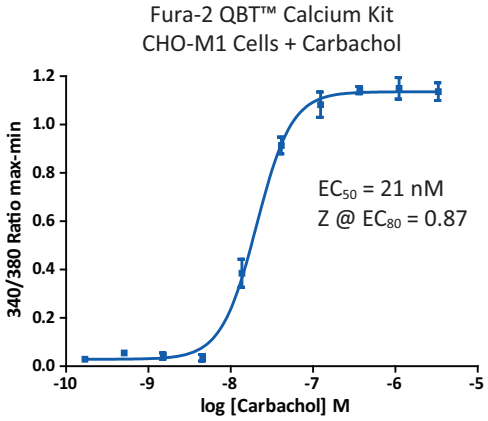
Data Analysis Examples

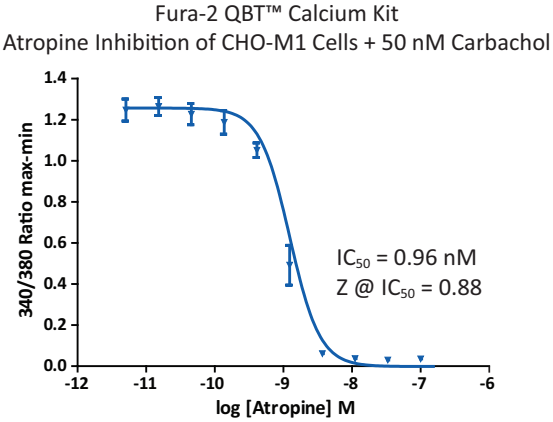
CHO-M1 Cells + ATP



In this assay, 10000 CHO M1 cells per well were plated in a black-walled, clear-bottom microplate overnight. On the day of the assay, the dye was made up following the directions listed in the protocol section. Equal volumes of dye were added to each well and the plate was incubated for 1 hour at 37°C, 5% CO_2. The assay results were read kinetically on the FLIPR Tetra instrument as ATP was added to stimulate the P2Y2 receptor. ScreenWorks® Software was used to calculate the ratio of the 340/380 nm wavelengths. The EC_{50} is 58 nM and the Z factor at EC_{80} is 0.79.

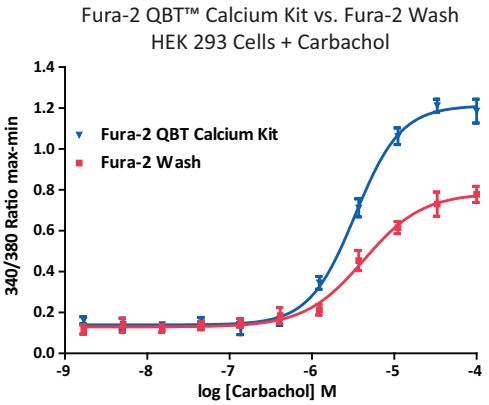
CHO-M1 Cells + Carbachol





In this assay, CHO-M1 cells with a transfected muscarinic M1 receptor were plated overnight and incubated with dye for one hour. Carbachol was used to generate a CRC as seen in the figure on the left. The EC_{50} value was 21 nM and in range with reported EC_{50} values. The Z factor at EC_{80} concentration was 0.87. In the figure on the right, 50 nM Carbachol was used as agonist vs. an antagonist CRC of Atropine. ScreenWorks Software was used to calculate the ratio of the 340/380 nm wavelengths. The IC_{50} value is 0.96 nM and the Z at IC_{50} value is 0.88.

HEK 293 Cells + Carbachol



	Fura-2 QBT	Fura-2 Wash
EC_{50} (µm)	3.3	4.3
Z @ EC_{80}	0.77	0.6

In this assay, two different assay methods are compared. HEK 293 cells were plated overnight in a 384-well black-walled, clear-bottom microplate. Cells were incubated with dye for one hour at 37°C, 5% CO_2. The endogenous Muscarinic M3 receptor was stimulated by Carbachol. The results from the Fura-2 QBTCalcium Kit are compared to the results from the traditional Fura-2 wash method. ScreenWorks Software was used to calculate the ratio of the 340/380 nm wavelengths. The signal window is larger with the Fura-2 QBT Calcium Kit. The Z factor at EC_{80} for the Fura-2 QBT Calcium Kit is also higher than the wash assay.

Chapter 8: Luminescence Assay Protocol

This protocol includes the following information:

Note: The references to the instrument assume the ICCD camera or HS EMCCD camera option is installed along with the Cell Suspension option.

Required Materials

The following materials are required for running a luminescence assay:

Table 8-1: Cell Preparation for Aequorin Assays

Item	Source
FLIPR Penta System with ICCD camera option or HS EMCCD camera, in luminescence mode, and Cell Suspension option	Molecular Devices
Clear, flat-bottom, black wall 96-, 384-, 1536-well plates	See Consumables and Accessories on page 79
Clear polypropylene source plates (for adherent protocols)	See Consumables and Accessories on page 79
Black FLIPR Penta System pipette tips, 96- or 384-well or 1536 tip block and gaskets	See Consumables and Accessories on page 79
Test compounds	Specific to receptor
Cells in suspension	Optional
Coelenterazine	Specific to Photoproteins
Growth medium	Major Laboratory Supplier (MLS)
Incubator	MLS
Centrifuge	MLS
Pipettor and sterile tips suitable for use with microplates	MLS

Experimental Protocol

Cell Densities

A variety of cell handling conditions may be adopted at your discretion based on standard operating procedures in the laboratory. Non-adherent cells are typically plated on the day of experiment on a coated plate (for example, poly-d-lysine or collagen) to assist cell adherence to the plate bottom. We recommend you then centrifuge the plates at 100 g for up to 4 min with the brake off. Adherent cells are seeded the day prior to an experiment and incubated in a 5% CO_2, 37°C (98.6°F) incubator overnight. To create an 80%–90% confluent cell monolayer, it is recommended the cells be seeded at densities shown in the table below. For suspension experiments, cells are loaded into a cell flask on the day of the experiment and are pipetted via the instrument into the read plate, so the flask concentration as well as the volume pipetted controls the final concentration.

The recommended concentrations are listed in the following table:

Cell Type (cells/well)	96-Well Plate (100 µL growth medium)	384-Well Plate (25 µL growth medium)	1536-Well Plate (2–3 µL growth medium)
Adherent cells	5,000–50,000	1,250–15,000	1,000–2,500
Non-adherent cells	40,000–100,000	10,000–60,000	2500–-15,000
Suspension cells	10,000–40,000	2,500–10,000	1,000–5,000

Coelenterazine Loading For Adherent Assays

To load the cells for an adherent assay:

1. Before the day of the assay, remove culture medium from wells.
2. Wash cells in 'BSA Medium' containing DMEM/HAM's F12 with HEPES, without phenol red, catalog# 11039-021 (Invitrogen) + 0.1% BSA.
3. In reduced lighting conditions, make up a solution of coelenterazine appropriate for your cell type, in 'BSA Medium' and add a volume of 100 µL (96-well plates), 25 µL (384-well plates), or 3 µL (1536-well plate) per well. Final concentration in the well should be 5–10 µM.
4. Incubate the cells for 4–6 hours at room temperature (22°C/71.6°F or lower). Cover the plates with aluminum foil to protect from light and to avoid degradation of coelenterazine.

Coelenterazine Loading for Suspension Cell Assays

To load the cells for a suspension assay:

1. Before the day of the assay, remove the selection antibiotics from cells.
2. On the day of the assay, wash cells in seeding flask with 20 mL DPBS (without Calcium and Magnesium).
3. Dislodge the cells by adding 15 mL Versene, incubating for 5 minutes in 37°C (98.6°F) 5% CO_2 and immediately adding 20 mL of growth media without selection antibiotics.
4. Centrifuge in 50 mL tube @ 1000 RPM or 168 g for 2.5–3 minutes.
5. With supernatant removed and cells re-suspended in 40 mL BSA-medium, count cells and adjust density to 5.0 x 10^6 cells/mL.
6. Under reduced lighting conditions, recommended type of coelenterazine should be added to reach a final concentration of 5 µL.
7. Cells should be placed in 15 mL aluminum wrapped tubes on rotating wheel (~7–8 RPM) at room temp (below 22°C/71.6°F) for 4 hours to overnight.
8. After incubation, cell density should be adjusted while protecting from light and cells should be placed in the cell stir flask and stirred on FLIPR Penta System at speed 6.
9. Cells should spin protected from light for 1–2 hours prior to the start of the assay.

Preparing the Cell Reservoir

To prepare the cell reservoir for a luminescence assay:

1. The Cell Reservoir must have been cleaned at the end of the previous day's assay. If this was done, rinse with DI H_2O, several times.
2. If the Cell Reservoir was not cleaned after previous assay, follow instructions for making AquaMax Sterilant Kit Procedure (Cat# R8156, Molecular Devices). See Consumables and Accessories on page 79.

3. Fill a flask with the sterilant solution and run three or four Cell Reservoir wash cycles including a two minute soak (Hold Time = 120 seconds).
4. Physically wipe the Cell Reservoir with Kimwipes moistened in DI water.
5. Rinse the Cell Reservoir five times with DI water and follow the steps for preparation in #6.
6. Before the start of the assay, Cell Reservoir and lines to and from spinner flask should be rinsed 5X each with the following:
   - 70% Ethanol
   - Endotoxin-free DI water
   - Media containing BSA-Medium 0.1% BSA
7. Before the start of screening, insert the hard plastic tubes from the Cell Flask into the lines running from the 8-way valve by pinching the lines and sliding the tubing inside.
8. Cycle the cells through to the Cell Reservoir two to three times before starting the assay.
9. Use two or three plates before screening and ensure that the camera settings are within range of the signal.

If cells are recirculated back to the cell flask, it is strongly recommended that the tips be washed each time an assay is run to reduce risk of contamination of the cells by toxic or antagonist compounds.

Optimizing an Assay

The majority of the FLIPR Penta System assay optimization is related to cell treatment before and during the assay. Cell and assay conditions to check include the following:

- Cell Culture
- Cell Seeding
- Loading Coelenterazine
- Source Plate on page 75

Cell Culture

Conditions to check:

- Cell passage number.
- Cell growth conditions, for example confluency in flasks and/or in wells.
- Expression induction time for transfected cells—concentration of selection antibiotics and overall cell viability.

Cell Seeding

Conditions to check:

- Seeding density. Do not allow cells to become over-confluent.
- Type of black, clear-bottom white or clear plate.
- Volume of growth medium in each well.
- Growth medium replacement during seeding period.

Loading Coelenterazine

Conditions to check:

- Seeding density. Do not allow cells to become over-confluent.
- Type of black, clear-bottom white or clear plate.
- Volume of growth medium in each well.
- Growth medium replacement during seeding period.

Source Plate

Conditions to check:

- Type of buffer used should match coelenterazine buffer.
- For suspension assay, prepare 2x compound directly in read plate.
- For adherent assays, prepare a polypropylene plate with 2x compound.
- Volume of fluid in source plate in relation to required transfer volume and plate dead volume.

Running a Luminescence Assay on a FLIPR Penta Instrument

Luminescence assays require no excitation source, but do require optimal instrument conditions to collect the maximum amount of light emitted from the assay. These settings are specific to the ICCD camera and HS EMCCD camera in luminescence mode camera option.

In order to provide optimal light collection by the FLIPR Penta System, we recommend you leave an empty emission filter position in the filter slider to run aequorin. In addition, you must calibrate the instrument to use no LEDs and emission filters. This state is identified as the NONE/NONE Excitation/Emission Wavelengths. Detailed instructions outlining the removal of the emission filters and calibration are found in the *FLIPR Penta High-Throughput Cellular Screening System User Guide*.

Recommended Settings for the FLIPR Penta Instrument

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

Recommended experiment setup parameters include the following:

Parameter	ICCD Camera	HS EMCCD Camera
Read Mode	Luminescence	Luminescence
Camera Mode	NA	Sensitivity
Excitation Wavelength	None	None
Emission Wavelength	None	None
Camera Gain[1]	70,000–280,000	323–500
Exposure Time[1] (s)	0.53	1
LED Excitation Intensity	NA	0.5 -1.0
Gate Open	100%	NA
Read Interval (s)	1	1.1
Reads before pipetting	5	5-10
Reads during first interval	50–90	50–90
Save Assay Images	Possible, but creates very large files	
1. Can be adjusted to compensate for cell brightness or saturation problems.		

Fluid Transfer Settings—Aspirate

Parameter	Suspension Setting (pipette cells)	Adherent Settings (pipette compound)
Cell Flask Spinning Rate	Manual setting, Speed 6	NA
Fluid Transfer Type	Single Aspirate, Single Dispense	Single Aspirate, Single Dispense
Source Plate Fill Reservoir speed Drain Reservoir speed Drain destination	defaultcellres384 5 4 Cell flask	Source Plate 2 NA NA NA
Aspiration Volume	25 µL	25 µL
Aspiration Height	70 µL	5 µL
Aspiration Speed	20 µL/s	20 µL/s
Tip Up Speed	10 mm/s	10 mm/s
Hold Volume	0 µL	0 µL

Fluid Transfer Settings—Dispense

Parameter	Suspension Setting	Adherent Settings
Target Plate	Read Plate	Read Plate
Volume	25 µL	25 µL
Height	15 µL	30 µL
Speed	30 µL/s	20 µL/s
Removal Speed	10 mm/s	10 mm/s
Expel Volume[1]	0 µL	0 µL
Pause in Well	None	None
Hold pipettor during dispense	None	None
1. A hold or expel volume will introduce air bubbles into the well causing false spikes in signal.		

Parameter	Setting
Reduction Type	Area under the curve, from or shortly after addition point to the end of the assay
Correction	Subtract bias based on Sample 1

Post Assay Cell Suspension System Cleaning

CAUTION! The cell suspension system should never be left without cleaning at the end of an assay day or between assays with different cells.

To clean the cell suspension system:

1. Remove cell spinner flask and insert tubing from flask into deionized (DI) water solution.
2. Insert return line tubing to a waste flask making sure that the tubing does not reach into the waste fluid. It will need to be left as clean as the inlet tubing.
3. Perform a manual reservoir wash with water and follow by wiping out the reservoir with a Kimwipe.
4. Build a FLIPR Penta System protocol to wash the reservoir and all tubing in the following order:
   - 5 times with Endotoxin-free DI water.
   - 5 times with Cytoclense Sterilant wash and soak (2 minutes).
   - 5 times with Endotoxin-free DI water.
   - 5 times with 70% Ethanol and leave system and all tubing dry.
5. The cell flask also must be washed each time after use:
   - Rinse flask, spinner, caps, and all tubing with Endotoxin-free DI water.
   - Use glassware detergent safe for use with cell culture vessel.
   - Scrub with bottle brush and flush lines.
   - Rinse with Endotoxin-free DI water.
   - Soak flasks in Cytoclense sterilant.
   - Rinse with Endotoxin-free DI Water.
   - Rinse with 70% Ethanol and leave all components to air dry or bake in oven.
   - In addition, reservoir and spinner flask may be autoclaved.

Appendix A: Consumables and Accessories

This following catalogs consumables used with the FLIPR Penta System, including:

System Accessories

Optional Software Modules

Supplier: Molecular Devices, call +1-800-635-5577.

Table A-1: Optional Software Modules

Item	Part Number
ScreenWorks® Peak Pro™ 1.0 Software (For ScreenWorks Software Versions 3.2 and 4)	5016900
ScreenWorks® Peak Pro™ 1.0 Software (For ScreenWorks Software Version 5)	5075630
ScreenWorks® Peak Pro™ 2.0 Software (For ScreenWorks Software Version 5)	5074843

Field Installations

Supplier: Molecular Devices, call +1-800-635-5577.

Table A-2: Field Installations Kits

Item	Part Number
FLIPR® Camera Conversion Kit, ICCD or EMCCD to HS EMCCD	5069580
FLIPR® Cell Suspension, Field Installation Kit	0310-5339
Windows 10 Host PC with ScreenWorks® Software Version 5 for ICCD Systems	5078498
Windows 10 Host PC with ScreenWorks® Software Version 5 *for select** EMCCD Systems	5075062
Windows 10 Host PC with ScreenWorks® Software Version 4.2 *for select** EMCCD Systems	5074979
FLIPR® Cycler	5074848
* Contact Molecular Devices to identify applicable kit	

Pipetting Tips and 1536 Pipetting Accessories

Supplier: Molecular Devices, call +1-800-635-5577.

Table A-3: Pipetting Consumables

Item	Part Number
FLIPR® pipette tips, black, non-sterile, 96-well, 50 racks/case	9000-0762
FLIPR® pipette tips, clear, non-sterile, 96-well, 50 racks/case	9000-0761
FLIPR® non-sterile, black, 384-well, 50 racks/case	9000-0764
FLIPR® pipette tips, clear, non-sterile, 384-well, 50 racks/case	9000-0763
FLIPR® 1536 tip gasket, nonsterile, 40 racks/case	9000-0746
FLIPR® 1536 tip block	0200-6112
[1]FLIPR® Pin Tool, 384	5075203
[1]FLIPR® Pin Tool, 1536	5075194
1. Contact your local Molecular Devices Sales Representative for details regarding this configure-to- order item.	

Pipettor Heads

Supplier: Molecular Devices, call +1-800-635-5577.

Table A-4: Pipettor Head Kits

Item	Part Number
FLIPR® Pipettor Head Kit, 96	0200-6071
FLIPR® Pipettor Head Kit, 384	0200-6072
FLIPR® Pipettor Head Kit, 1536	0200-6073
[1]FLIPR® Pin Tool Kit, 384 and/or 1536	FLIPR PIN TOOLS OC
1. Contact your local Molecular Devices Sales Representative for details regarding this configure-to- order item.	

Optics Consumables

Supplier: Molecular Devices, call +1-800-635-5577.

Table A-5: Optics Kits

Item	Part Number
FLIPR® Calcium Optics Kit	0200-6206
FLIPR® Membrane Potential Optics Kit	0200-6207
FLIPR® Voltage Sensor Probes (VSP) Optics Kit	0200-6208

Table A-5: Optics Kits (continued)

Item	Part Number
FLIPR® Fura-2 Optics Kit	0200-6271
FLIPR® LED Module, 335–345 nm (UV)	0200-6272
FLIPR® LED Module, 360–380 nm	0200-6178
FLIPR® LED Module, 380–390 nm (UV)	0200-6273
FLIPR® LED Module, 390–420 nm (VSP)	0200-6135
FLIPR® LED Module, 420–455 nm	0200-6148
FLIPR® LED Module, 470–495 nm (Calcium)	0200-6128
FLIPR® LED Module, 495–505 nm	0200-6175
FLIPR® LED Module, 510–545 nm (Membrane Potential)	0200-6127
FLIPR® LED Module, 610–626 nm	0200-6150
FLIPR® Emission Filter, 400–460 nm	0200-6213
FLIPR® Emission Filter, 440–480 nm (VSP)	0200-6205
FLIPR® Emission Filter, 475–535 nm	0200-6211
FLIPR® Emission Filter, 515–575 nm (Calcium)	0200-6203
FLIPR® Emission Filter, 526–586 nm	0200-6212
FLIPR® Emission Filter, 565–625 nm (Membrane Potential and VSP)	0200-6204
FLIPR® Emission Filter, 646–706 nm	0200-6214
FLIPR® Custom Filter Set (3)	0200-6221
FLIPR® Single Custom Filter Holder (1)	0200-6276

Cell Reservoir Consumables

Supplier: Molecular Devices, call +1-800-635-5577.

Table A-6: Cell Reservoir Consumables

Item	Part Number
FLIPR® Cell Suspension Reservoir	0200-6222
FLIPR® 250 mL Flask Assembly	0200-6223
FLIPR® 500 mL Flask Assembly	0200-6224
FLIPR® 1 L Flask Assembly	0200-6225
FLIPR® 3 L Flask Assembly	0200-6226
FLIPR® 1 L Buffer Bottle	0200-6227

Plates

The following conforming plates, can be purchased from Molecular Devices.

96-Well Read Plates

Table A-7: 96-Well Read Plates

96-Well Read Plates	Part Number	Suggested Supplier	Phone Number
Black, clear, tissue culture treated, sterile	353948	Becton Dickinson	+1-800-343-2035
	3603	Corning/Costar	+1-800-492-1110
	655090	Greiner (distributed by E&K)	+1-408-378-2013
	165305	Nalge/Nunc	+1-800-766-7000
Black, clear, tissue culture treated, sterile, poly-D-lysine coated	356640	Becton Dickinson	+1-800-343-2035
	3667	Corning/Costar	+1-800-492-1110
Black, clear, tissue culture treated, sterile, collagen coated	356649	Becton Dickinson	+1-800-492-1110
White, clear, tissue culture treated, sterile	353947	Becton Dickinson	+1-800-343-2035
	3903	Corning/Costar	+1-800-492-1110
	655098	Greiner (distributed by E&K)	+1-408-378-2013
	165306	Nalge/Nunc	+1-800-766-7000

96-Well Read Plate Masks Made by Molecular Devices

Table A-8: 96-Well Read Plate Masks Made by Molecular Devices

96-Well Read Plate Masks	Part Number	Plate Manufacturer
96-well slit mask	0200-6143	Becton Dickinson
	2300-1362	Corning/Costar
	0200-6142	Nalge/Nunc
	0200-6144	Greiner

96-Well Source Plates

Table A-9: 96-Well Source Plates

96-Well Source Plates	Part Number	Suggested Supplier	Phone Number
V-bottom plate, 96-well	353263 polypropylene	Becton Dickinson	+1-800-343-2035
	651201 polypropylene	Greiner (distributed by E&K)	+1-408-378-2013
	249944	Nalge/Nunc	+1-800-766-7000
U-bottomed plate, 96-well	351190 polypropylene	Becton Dickinson	+1-800-343-2035
	3365 polypropylene	Corning/Costar	+1-800-492-1110
	267245	Nalge/Nunc	+1-800-766-7000
	650201 polypropylene	Greiner (distributed by E&K)	+1-408-378-2013
Deep-well plate, 96-well	353966 polypropylene	Becton Dickinson	+1-800-343-2035
	780270 polypropylene	Greiner (distributed by E&K)	+1-408-378-2013
	278752 polypropylene	Nalge/Nunc	+1-800-766-7000

384-Well Read Plates

Table A-10: 384-Well Read Plates

384-Well Read Plates	Part Number	Suggested Supplier	Phone Number
Black, clear, tissue culture treated, sterile	353262	Becton Dickinson	+1-800-343-2035
	3712	Corning/Costar	+1-800-492-1110
	781091	Greiner with lids (distributed by E&K)	+1-408-378-2013
	MGB101-1-2	Matrical	+1-509-343-6225
	MGB101-1-3 0.17 mm-thick glass	Matrical	+1-509-343-6225
	142761	Nalge/Nunc	+1-800-766-7000
Black, clear bottom, tissue culture treated, sterile, poly-D-lysine coated	356663	Becton Dickinson	+1-800-343-2035
	3664	Corning/Costar	+1-800-492-1110

Table A-10: 384-Well Read Plates (continued)

384-Well Read Plates	Part Number	Suggested Supplier	Phone Number
Black, clear, tissue culture treated, sterile, collagen coated	356667	Becton Dickinson	+1-800-343-2035
White, clear, tissue culture treated, sterile	353963	Becton Dickinson	+1-800-343-2035
	3707	Corning/Costar	+1-800-492-1110
	781098	Greiner (distributed by E&K)	+1-408-378-2013
	142762	Nalge/Nunc	+1-800-766-7000
Low volume black, clear, tissue culture treated, sterile	3542	Corning/Costar	+1-800-492-1110
	788092	Greiner (distributed by E&K)	+1-408-378-2013

384-Well Source Plates

Table A-11: 384-Well Source Plates

384-Well Source Plates	Part Number	Suggested Supplier	Phone Number
Flat bottom plate	353265 polypropylene	Becton Dickinson	+1-800-343-2035
	3702 polystyrene	Corning/Costar	+1-800-492-1110
	781201 polypropylene	Greiner (distributed by E&K)	+1-408-378-2013
	265496 polystyrene	Nalge/Nunc	+1-800-766-7000
U/V-bottom plate	3657 polypropylene	Corning/Costar	+1-800-492-1110
	781280 polypropylene	Greiner (distributed by E&K)	+1-408-378-2013
	264573 polypropylene	Nalge/Nunc	+1-800-766-7000
Deep-well plate	353996 polypropylene	Becton Dickinson	+1-800-343-2035
	3965 polypropylene	Corning/Costar	+1-800-492-1110
	781270 polypropylene	Greiner (distributed by E&K)	+1-408-378-2013
	MP102 polypropylene	Matrical	+1-509-343-6225

1536-Well Read Plates

Table A-12: 1536-Well Read Plates

1536-Well Read Plates	Part Number	Suggested Supplier	Phone Number
Black/Clear, tissue culture treated, sterile	783092	Greiner with lids (distributed by E&K)	+1-408-378-2013
White/Clear, tissue culture treated, sterile	781098	Greiner (distributed by E&K)	+1-408-378-2013

1536-Well Source Plates

Table A-13: 1536-Well Source Plates

1536-Well Source Plates	Part Number	Suggested Supplier	Phone Number
Deep-well plate	782270 polypropylene	Greiner (distributed by E&K)	+1-408-378-2013

Source Reservoirs

Table A-14: Source Reservoirs

Source Reservoirs	Part Number	Suggested Supplier	Phone Number
Omni tray	2428110 polystyrene	Nalge/Nunc	+1-800-786-7000

Assays

Calcium Flux Consumables and Accessories

Table A-15: Calcium Flux Consumables and Accessories

Item	Part Number	Suggested Supplier	Phone Number
FLIPR® Calcium 6 Assay Kit: Bulk Kit Explorer Kit Express Kit	 R8190 R8191 R8195	Molecular Devices	+1-800-635-5577 +1-408-747-1700
FLIPR® Calcium 6 QF Assay Kit: Bulk Kit Explorer Kit Express Kit	 R8192 R8193 R8196	Molecular Devices	+1-800-635-5577 +1-408-747-1700
FLIPR® Calcium 5 Assay Kit: Bulk Kit Explorer Kit Express Kit	 R8186 R8185 R8187	Molecular Devices	+1-800-635-5577 +1-408-747-1700
FLIPR® Calcium 4 Assay Kit: Bulk Kit Explorer Kit Express Kit	 R8141 R8142 R8143	Molecular Devices	+1-800-635-5577 +1-408-747-1700
FLIPR® Calcium 3 Assay Kit: Bulk Kit Explorer Kit Express Kit	 R8090 R8091 R8108	Molecular Devices	+1-800-635-5577 +1-408-747-1700
FLIPR® Cardiocytoxicity Assay Kit: Bulk Kit Explorer Kit	 R8211 (10 plates) R8210 (2 plates)	Molecular Devices	+1-800-635-5577 +1-408-747-1700
FLIPR® Fura-2 QBT Calcium Assay Kit: Bulk Kit Explorer Kit	 R8197 R8198	Molecular Devices	+1-800-635-5577 +1-408-747-1700
FLIPR® Penta System Calcium Optics Kit: LED Module, 470-495 nm Emission Filter, 515-575 nm	0200- 6206	Molecular Devices	+1-800-635-5577 +1-408-747-1700

Table A-15: Calcium Flux Consumables and Accessories (continued)

Item	Part Number	Suggested Supplier	Phone Number
FLIPR® Penta System Fura-2 Optics Kit: LED Module 333-345 nm LED Module 380-390 nm Emission Filter 475-535nm	0200-6271	Molecular Devices	+1-800-635-5577 +1-408-747-1700
Hank's Balanced Salt Solution (10X stock)	14065- 056	Thermo Fisher	1-800-828-6686
HEPES buffer solution 1X	9319	Irvine Scientific	+1-800-437-5706
Probenecid, crystalline	P8761	Sigma	+1-800-325-3010
Carbachol (receptormediated positive control)	C4382		
UTP, Na salt (receptormediated positive control)	U6625		
Ionomycin (positive control)	407950	CalBiochem	+1-800-854-3417

Membrane Potential Assay Kit Consumables and Accessories

Table A-16: Membrane Potential Assay Kit Consumables and Accessories

Item	Part Number	Suggested Supplier	Phone Number
FLIPR® Membrane Potential Assay Kit	R8034 (BLUE) R8123 (RED)	Molecular Devices	+1-800-635-5577 +1-408-747-1700
FLIPR® Penta System Membrane Potential Optics Kit: FLIPR® Penta System LED Module, 510–545 nm FLIPR® Penta System Emission Filter, 565–625 nm	0200-6207	Molecular Devices	+1-800-635-5577 +1-408-747-1700
Hank's Balanced Salt Solution (10X stock)	14065-056	Thermo Fisher	1-800-828-6686
HEPES buffer solution 1X	9319	Irvine Scientific	+1-800-437-5706
Carbachol (receptor-mediated positive control)	C4382	Sigma	+1-800-325-3010

Potassium Assay Kit Consumables and Accessories

Table A-17: Potassium Assay Kit Consumables and Accessories

Item	Part Number	Suggested Supplier	Phone Number
FLIPR® Potassium Assay Kit Evaluation Kit Explorer Kit Bulk Kit	 R8330 R8222 R8223	Molecular Devices	+1-800-635-5577 +1-408-747-1700
FLIPR® Penta System Calcium Optics Kit: FLIPR® Penta System LED Module, 470-495 nm FLIPR® Penta System Emission Filter, 515-575 nm	0200-6206	Molecular Devices	+1-800-635-5577 +1-408-747-1700
Hank's Balanced Salt Solution (10X stock)	14065-056	Thermo Fisher	1-800-828-6686
HEPES buffer solution 1X	9319	Irvine Scientific	+1-800-437-5706
DMSO	D8418	Sigma	+1-800-325-3010
Probenecid	P8761	Sigma	+1-800-325-3010

Recommended Assay Equipment and Supplies

The following recommended assay equipment and supplies are not available through Molecular Devices:

- 5 mL, 10 mL, 25 mL sterile serological pipettes
- Rechargeable pipettor for 2–25 mL pipettes
- Sterile tissue culture water
- Gloves
- Culture medium to grow cells
- EDTA and Trypsin/EDTA to lift cells
- Hemacytometer and counter
- Sterile test tubes 15 mL and 50 mL or smaller tubes for compounds dilutions
- 1 N NaOH solution to dissolve probenecid

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