Product Insert

FLIPR® Calcium 3 Assay Kit

Product # R8091 (Explorer Kit)
R8090 (Bulk Kit)
R8108 (Express Kit)

Introduction

About the FLIPR Calcium 3 Assay Kit

The Calcium 3 Assay Kit from Molecular Devices Corporation provides a fast, simple and reliable fluorescence-based assay for detecting changes in intracellular calcium. With this kit, calcium assays on FLIPR or FlexStation® become a mix-and-read procedure in which cells are incubated with the kit reagents for one hour and transferred directly to FLIPR or FlexStation for evaluation. There are no intermediate wash-steps involved.

Conventional calcium mobilization protocols are multi-step procedures which begin by pre-washing the cells, loading them with a calcium indicator (e.g.: Fluo-3 or Fluo-4), followed by extensive cell washing prior to running the assay. Several problems are routinely encountered when washing dye-loaded cells, all of which may add to experimental variability.

Problems in a conventional assay protocol include:

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

Molecular Devices developed the FLIPR Calcium 3 Assay Kit to eliminate the cause of data variability and reduce the number of steps in the conventional wash protocol using Fluo-3 or Fluo-4. It offers additional advantages over the FLIPR Calcium and Calcium Plus Assay Kits.

Advantages of the FLIPR Calcium 3 Assay Kit include:

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

Applications

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

Materials

Kit Components

Table 1: FLIPR Calcium 3 Assay Kit (P/N R8091, R8090, R8108) contents.

Reagent	Description

R8091 (Explorer Kit)	• 10 vials Component A
	• 1 bottle Component B
	  • 1X Hank's Balanced Salt solution
	  • pH 7.4, 20 mM Hepes buffer
	• The entire kit is sufficient for ten 96-, 384-, or 1536-well plates. Each vial is sufficient for assaying one 96-, 384-, or 1536-well plate.

R8090 (Bulk Kit)	• 10 vials Component A • The entire kit is sufficient for one hundred 96-, 384-, or one hundred fifty 1536-well plates. Each vial is sufficient for assaying ten 96-, 384-well, or fifteen 1536-well plates.
R8108 (Express Kit)	• 2 vials Component A • The entire kit is sufficient for one hundred 96-, 384-, or one hundred fifty 1536-well plates. Each vial is sufficient for assaying fifty 96- or 384-well, or seventy-five 1536-well plates.

Materials Required but Not Provided

Table 2: Reagents and supplies

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

Storage and Handling

On receipt of the FLIPR Calcium 3 Assay Kit, store contents at –20°C. Under these conditions the reagents are stable for six months in the original packaging.

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

Calcium 3 Assay Kit Experimental Protocol

A. Cell Handling

The FLIPR Calcium 3 Assay Kit is designed to work with many cell types, both adherent and non-adherent. We recognize that a variety of cell handling conditions might be adopted at the discretion of the user, based on standard operating procedures in the laboratory. In this section, we provide guidelines on how to prepare the cells for use with the assay kit.

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

Table 3: Suggested plating volumes and seeding densities

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

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

B. Preparation of Loading Buffer

The following procedure is designed for preparation of the Loading Buffer per vial of the **Explorer Kit (R8091),** the **Bulk Kit (R8090),** or the **Express Kit (R8108)**.

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

2. Remove one vial of FLIPR Calcium 3 Assay Reagent **(Component A)** and equilibrate to room temperature.

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

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

Plate Format	Explorer Kit (R8091)	Bulk Kit (R8090)	Express Kit (R8108)
96- or 384-well	10 mL	10 mL	20 mL
1536-well	6.5 mL	10 mL	20 mL

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

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

Plate Format	Explorer Kit (R8091)	Bulk Kit (R8090)	Express Kit (R8108)
96- or 384- well	N/A	90 mL	480 mL
1536-well	N/A	55 mL	300 mL

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

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

C. Loading cells using Loading Buffer

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

   Note: Although Molecular Devices does not recommend washing cells before dye loading, growth medium and serum may interfere with certain assays or cause a fluorescence drop upon compound addition due to media autofluorescence. In this case, the supernatant can be aspirated and replaced with an equal volume of serum-free HBSS buffer before adding the Loading Buffer. Alternatively, cells can be grown in serum-free conditions.

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

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

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

D. Running the Calcium Mobilization Assay

FLIPR

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

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

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

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

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

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

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

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

FlexStation

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

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

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

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

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

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

4. Analyze the data using SOFTmax Pro.

Trouble-shooting Guide

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

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

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

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

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

Precipitation in the Reagent Buffer.
The FLIPR Calcium 3 Assay Kit is compatible with numerous buffers. Use buffers shown to work in previously established assays, if available.

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

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

Data Analysis

FLIPR Assay Examples

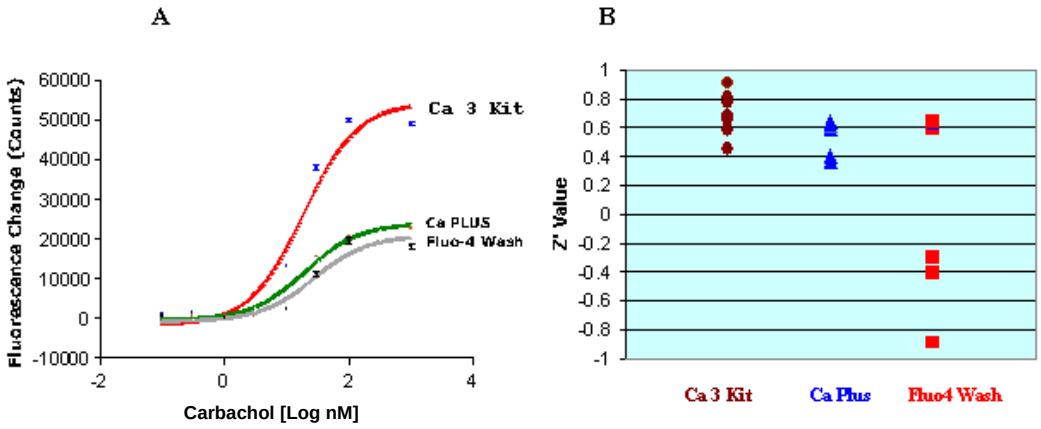

Figure 1.
A. CHO cells stably transfected with Muscarinic receptor 1 (CHO-M1) were seeded overnight in 100 µL per well of a 96-well Costar plate. Cells were incubated with 100 µL of the FLIPR Calcium 3 Assay Kit, Calcium Plus Assay Kit, or Fluo-4 (4 µM) for 1 h at 37ºC. Cells incubated with Fluo-4 were washed 3 times with 200 µL 1X HBSS buffer with 2.5 mM probenecid per well using an EMBLA 96/384 well washer (Molecular Devices). Then 1X HBSS buffer with 2.5 mM probenecid (200 µL/well) was added. Carbachol was added (50 µL/well) to achieve the final indicated concentration (n = 12/group).
B. Composition of data quality of calcium response in a GPCR cell line using the FLIPR Calcium 3 Assay Kit, FLIPR Calcium Plus Kit or Fluo-4 in a wash procedure. Each data point represents the Z'-factor for a GPCR agonist at its EC_{75} in all wells of a 96 well plate.

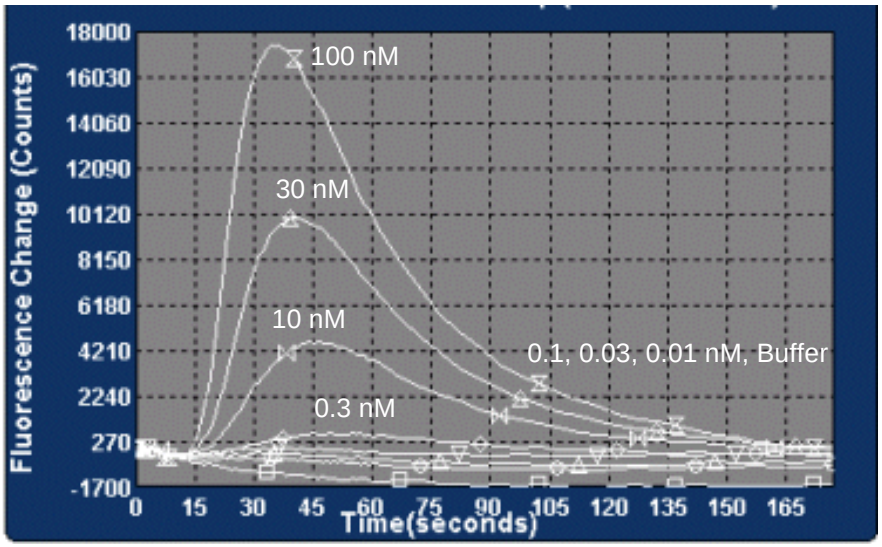

Figure 2. CHO cells stably transfected with CCR5 and Gqi5 were seeded overnight at 100 µL per well in a 96-well Poly-D-Lysine coated plate. Cells were incubated with 100 µL of Calcium 3 Assay Kit for 1 h at 37ºC. RANTES was added (50 µL /well) to achieve the final indicated concentration (n = 4) ($FLIPR^3$ screenshot).

FLIPR^{TETRA} 1536-well Assay Examples

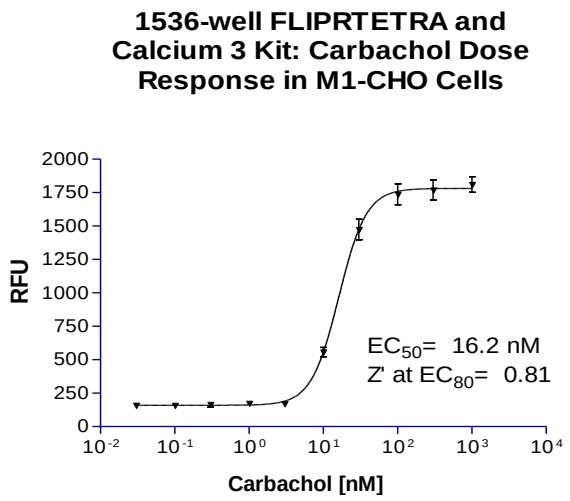

Figure 3. Carbachol dose response in CHO cells stably transfected with Muscarinic receptor 1 (CHO-M1). Cells were seeded overnight in 4 μL per well (2000 cells per well) in a 1536-well Greiner low-base plate. Cells were incubated with 2 μL of the Calcium 3 Assay Kit for 1 hour at 37ºC. Carbachol was added (1.0 μL /well) to achieve the final indicated concentration (n = 24).

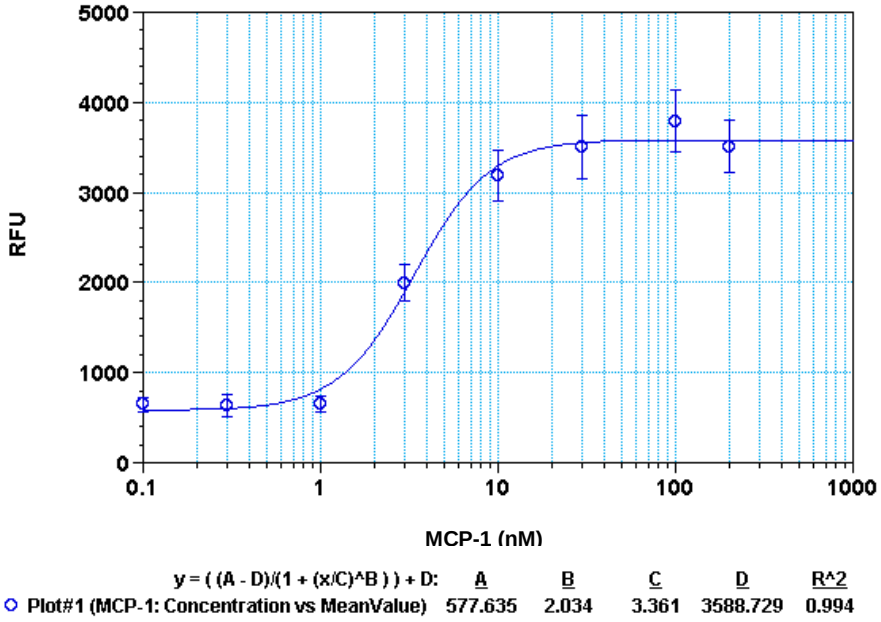

Figure 4. MCP-1 dose response. CHO cells transfected with CCR2B and Gα16 were seeded in Ham's F12 media + 1% FBS overnight in 4 μL per well in a 1536-well Greiner low-base plate. Cells were incubated with 2 μL of the Calcium 3 Assay Kit for 1 h at 37ºC. MCP-1 was added (1 μL/well) to achieve the final indicated concentration (n = 48-144).

FlexStation Assay Examples

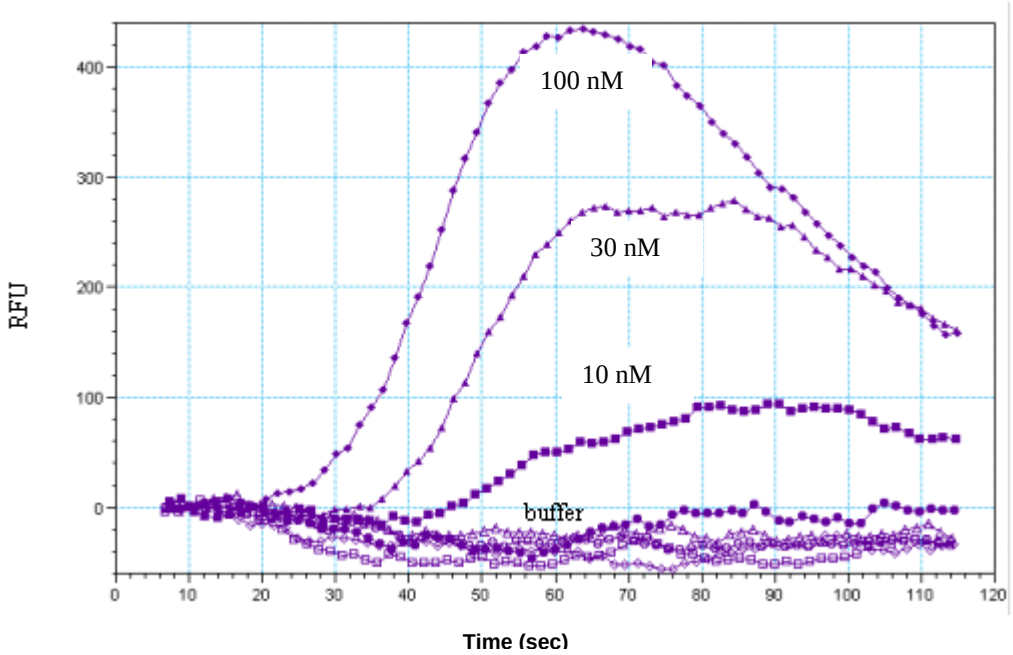

Figure 5. CHO cells stably transfected with CCR5 and $Ga_{qi}5$ were seeded overnight in 100 µL in a 96-well Poly-D-Lysine coated plate. Cells were incubated with 100 µL of FLIPR Calcium 3 Assay Kit for 1 h at 37°C. RANTES was added (50 µL/well) to achieve the final indicated concentration (n = 4) (FlexStation screenshot).

Appendix A AquaMax® DW4 protocols for dispensing into and aspirating from 1536-well plates

The Molecular Devices' AquaMax DW4 (DW4) is the preferred device for dispensing cells and bulk reagents in 1536-well formats. In addition, the DW4 can remove media prior to cell loading, an alternative that may be useful in situations where media or serum contributes to interferences in measuring calcium responses. Here are procedures for cleaning the DW4, for using it to dispense cells and reagents, and to aspirate off media. Please refer to your DW4 Operator's manual for instructions on operating the washer.

DW4 cleaning procedure before and after dispensing cells

To maintain sterility and to prevent clogging of the DW4, we recommend the following procedure before and after each daily use:

1. Clean dispense head(s) with 10% bleach (i.e.: 1:10 of standard 6.15% sodium hypochlorite bleach) 15 times
2. Clean dispense heads with DI water 15-30 times
3. Dry dispense head(s) with air 3 times
4. Clean reservoir bottle(s) 5 times with de-ionized water
5. Rinse reservoir bottle(s) 1 time with 70% Ethanol
6. Sterilize reservoir bottle(s). Bottles can be autoclaved. The lids can not be repeatedly autoclaved, but can be sterilized with 70% Ethanol

Alternatively, you may use the AquaMax Sterilant (R8156) kit for cleaning the DW4.

Setting up the 1536-well Parameters for the DW4

See the DW4 User Manual for details on how to set up a protocol. The following suggested parameters are intended for use with the 1536 Greiner low-base plate (catalog #783092).

1. Plate dimension set up	
Off set (A1):	7.87
Well to well center:	2.25
Height:	10.40
Well depth:	8.6
Max volume:	13.0
2. Dispense parameters	
Plate type:	1536 Greiner low based
Rows:	All
Volume dispensed:	4.0ul
Liquid factor:	0.95
3. Aspirate parameters	
Plate type:	1536 Greiner low based
Rows:	All
Velocity:	Medium speed
T (aspirate):	1 seconds
Probe height:	2 (mm)

Choosing DW4 file

When you are ready to use these programs, they are to be uploaded from computer to AquaMax DW4 file. Choose desired file and push START.

Protocol for dispensing cells

1. Clean the DW4 as described above.
2. Rinse reservoir bottle with sterile PBS before loading with cells.
3. Fill the desired reservoir with cells, allowing 3-10 mL extra for priming.
4. Be sure 1536 heads are attached to the DW4.
5. Select “Prime” on the DW4 front panel and choose the desired reservoir. Prime one time.
6. Select the desired dispense protocol on the DW4 panel (see section A. for volumes).
7. Insert a dummy plate and dispense 2-4 rows to make sure that all inlets are dispensing.
8. Insert the assay plate and press start.
9. Place the cell plates into the hood for about 30 minutes to decrease the edge effects.
10. Repeat steps 8 and 9 for each plate.
11. Place plates in incubator or desired location.
12. Clean the DW4 as described above.

Protocol for adding reagents to cells

This can be loading buffer (as in section B.4) or assay buffer

1. Clean the DW4 as described above.
2. Prepare loading buffer as described in step B.4. For 1536-well plates, the buffer volume is 65 mL (bulk kit R8090) or 320 ml (Express kit R8108) per vial, and if required, probenecid is added so that the final concentration in the well is 2.5 mM.
3. Rinse reservoir bottle with sterile PBS before filling with loading buffer.
4. Fill the desired reservoir with loading buffer, allowing 3-10 mL extra for priming.
5. Be sure 1536 heads are attached to the DW4.
6. Select “Prime” on the DW4 front panel and choose the desired reservoir. Prime one time.
7. Select the desired dispense protocol on the DW4 panel (see section C.1 for volumes).
8. Insert a dummy plate and dispense 2-4 rows to make sure that all inlets are dispensing.
9. Insert the assay plate and press start.
10. Repeat steps 8 and 9 for each plate.
11. Place plates in incubator or desired location.
12. Clean the DW4 as described above.

Protocol for aspiration of media

1. Clean the DW4 as described above.
2. Be sure the 1536 aspiration head is attached to the DW4.
3. Select “Clean” on the DW4 front panel to clean the aspiration head.
4. When prompted, insert a liquid tray with sterile PBS in it.
5. Press “Start” to clean the aspiration head.
6. Select the desired aspirate protocol on the DW4 panel.
7. Insert the assay plate and press start.
8. Repeat step 7 for each plate.
9. Place plates in incubator or desired location.
10. Clean aspiration head with water, a solution of 10% bleach, then water.

Product Use Limitations and Warranty

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

Each product is shipped with documentation stating specifications and other technical information. Molecular Devices products are warranted to meet or exceed the stated specifications. Molecular Devices’ sole obligation and the customer’s sole remedy are limited to replacement of the products free of charge in the event that the product fails to perform as warranted.

Molecular Devices Corporation makes no other warranties, either expressed or implied, including without limitation the implied warranties of merchantability and fitness for a particular purpose or use.

Molecular Devices Corporation is the exclusive licensee of a patented assay technology from Bayer A.G. (U.S. patent number 6,420,183, European patent number 0,906,572 and family members throughout the world). We are the only authorized provider of any assay incorporating enhancing, optimizing, quenching or masking agents as claimed in Bayer’s patented technology. The purchase of this assay kit from Molecular Devices Corporation includes a non-exclusive right to practice Bayer A.G., U.S. Patent 6,420,183, European Patent 0,906,572, and foreign counterparts thereof, in conjunction with and only in conjunction with the use of this assay kit.

Molecular Devices

Molecular Devices Corporation
3860 North 1st Street
San Jose, CA 95134 USA
Email: info@moldev.com
www.moleculardevices.com

Sales Offices

USA 800-635-5577 • **UK**+44-118-944-8000 • **Germany** +49-89-9620-2340 • **Japan** +06-6399-8211
Check our web site for a current listing of our worldwide distributors.

R3404 Rev. F 11/29/18