Fura-2 QBT™ Calcium Kit

The Fura-2 QBT™ Calcium Kits from Molecular Devices® provide a fast, simple, and reliable ratiometric fluorescence-based assay for detecting changes in intracellular calcium. The kits are based on the Fura-2 calcium indicator that permits ratiometric measurement of calcium concentrations. The Fura-2 QBT Calcium Kit uses the same proprietary quench technology used in the FLIPR® Assay Kits. Each kit provides mix-and-read procedures for calcium flux assays in which cells are incubated with the kit reagents for one hour and transferred directly to the FLIPR® Penta High-Throughput Cellular Screening System or FlexStation® 3 Multi-Mode Microplate Reader for evaluation. There are no intermediate wash-steps required with this kit.

Table A-1: Available Kits

Assay Kit	Explorer Kit	Bulk Kit
Fura-2 QBT™ Calcium Kit	R8197	R8198

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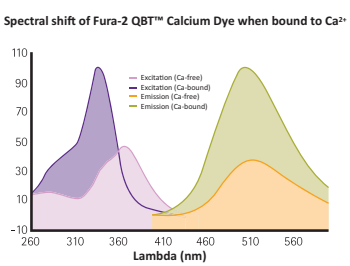
Chapter 1: About the Fura-2 QBT Calcium Kit

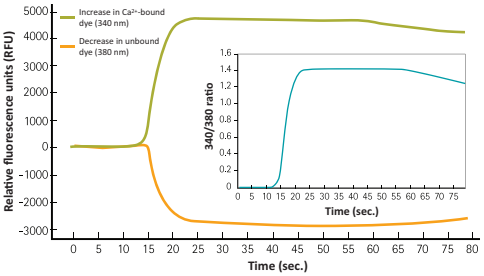
Molecular Devices calcium assays employ sensitive calcium indicators and a proprietary masking dye. The Calcium Kits contain Fura-2, acetoxymethyl (AM), a cell permeable calcium indicator that is ratiometric, and UV-light excitable. The Ca^{2+}-bound Fura-2 dye is excited at 340 nm, and the unbound form 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.

Chapter 2: Materials and Equipment

Kit Components

Table 2-1: 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 2-2: 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 contents at –20°C (-4°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

Compatible Molecular Devices Instruments

The Fura-2 QBT Calcium Kit is designed to be used with the following Molecular Devices instruments and their corresponding tips:

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

Chapter 3: Experimental Protocol

Quick Start Protocol

To run a 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 3-1 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 3-1: Suggested Plating Volumes and Seeding Densities

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

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 3-2.

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 3-2: 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 5. Assay development might be required to determine the best concentration.

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

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

Loading Cells Using Loading Buffer

To load cells:

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 Instrument

After incubation, transfer the microplates to the FLIPR 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 Tetra System or FLIPR Penta instrument with an EMCCD camera, adjust typical average baseline counts to a range from 800 RFU to 1500 RFU for each LED set. Keep the gain setting 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 Tetra System or 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 Instrument

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

Table 3-3: Experimental setup parameters for a 96-well or 384-well microplate on a FLIPR Tetra and 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

Table 3-3: Experimental setup parameters for a 96-well or 384-well microplate on a FLIPR Tetra and Penta instrument (continued)

Parameter	EMCCD Camera	HS EMCCD Camera	ICCD Camera
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

Running the Calcium Mobilization Assay on a FlexStation 3 Instrument

After incubation, transfer the microplate directly to the FlexStation 3 instrument assay plate carriage and run the assay.

In an individual well or column of wells, the calcium flux peak(s) should be complete within 1 to 3 minutes after addition. For an entire plate, however, the protocol will not complete until all chosen columns are finished. The assays are run one column at a time. Due to the time interval required to optimize reading of both wavelengths, Molecular Devices recommends that 8 wells per column be read during data acquisition.

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

Analyze the data using the SoftMax® Pro Software.

Recommended Settings for the FlexStation 3 Instrument

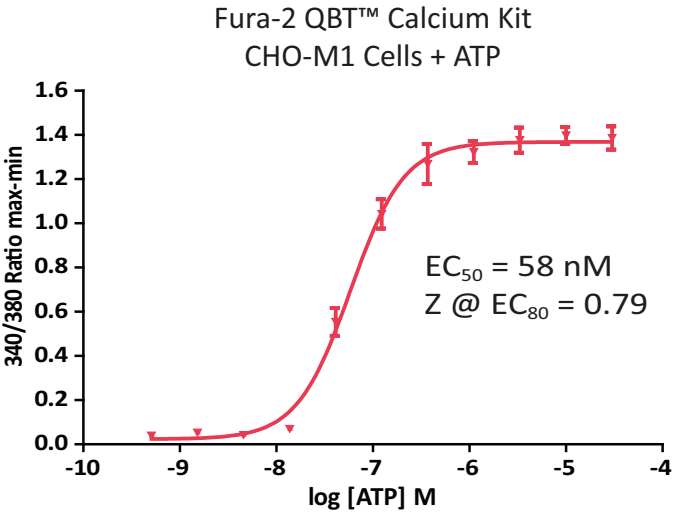
Before reading the microplate, set up the FlexStation 3 instrument using a SoftMax Pro Software protocol. The recommended experimental setup parameters are listed in Table 3-4.

Table 3-4: Experiment Setup Parameters for the FlexStation 3 Instrument

Parameter	96-well microplate	384-well microplate
Excitation Wavelength (nm)	340 380	340 380
Emission Wavelength (nm)	510	510
Automatic Emission Cut-Off (nm)	495	495
PMT Sensitivity	Medium (Readings: 3)	Medium (Readings: 3)
Pipette Height (µL)	230	40
Transfer Volume (µL)	50	12.5
Compound Concentration (Fold)	5X	5X
Addition Speed (Rate) Adherent Cells	3	3
Addition Speed (Rate) Non-Adherent Cells	1	1

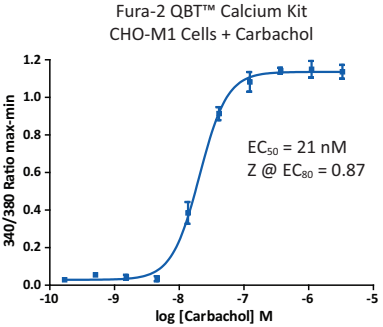
Chapter 4: Data Analysis Examples

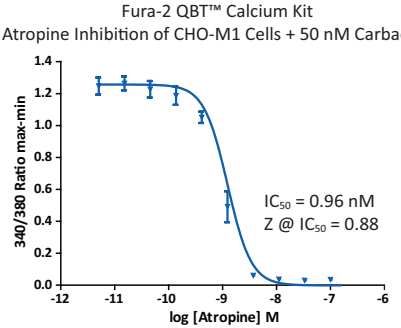
CHO-M1 Cells + ATP



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.

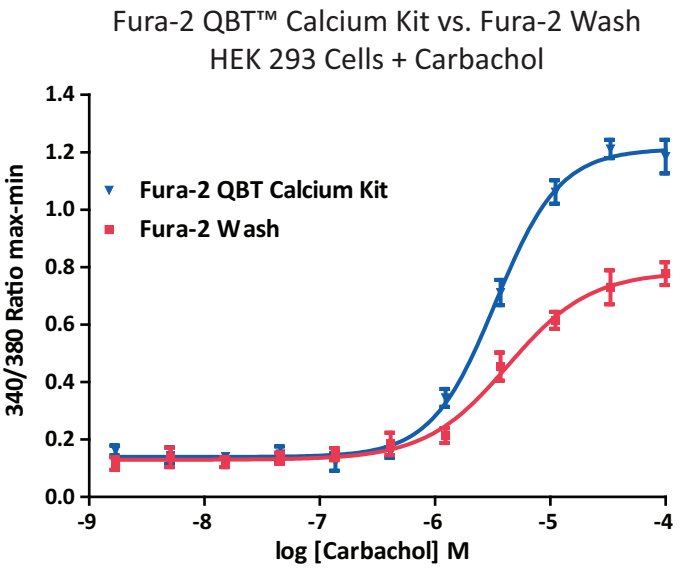
CHO-M1 Cells + Carbachol





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.

Obtaining Support

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

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

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

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