Fura-2 QBT ratiometric calcium kit, a novel homogeneous assay for measuring calcium flux assays on the FLIPR Tetra System and the FlexStation 3 Microplate Reader

Simon Lydford[1], Laurence, Monnet[1], Carole Crittenden[2], Yen-Wen Chen[2]
[1]Molecular Devices, 660-665 Eskdale Rd., Winnerish Triangle, Wokingham, Berkshire, RG415TS, UK
[2]Molecular Devices, LLC, 1311 Orleans Drive, Sunnyvale, CA 94089

Abstract

Receptor-ligand mediated change in the concentration of intracellular calcium is one of the most common signal transduction events measured in cell-based assays. Calcium mobilization assays monitoring Gq-coupled GPCR activity using single wavelength dyes such as Fluo-4, can on occasion miss responses due to interference from a number of factors; auto-fluorescent compounds excited at the same wavelength as the dye, large intracellular calcium concentration changes, the use of GFP tagged receptors or greater than desired well to well variability-especially when the wells need to be washed. Fura-2, being a dual wavelength calcium indicator, undergoes a spectral shift proportional to intracellular calcium concentration. As a result of the dual excitation, Fura-2 is typically not sensitive to the issues seen with most single wavelength dyes.

One of the main drawbacks of using Fura-2 dye in the traditional method is the required wash step prior to running the assay. Washing cell plates can cause well to well variability, requires a large volume of buffer, and lowers throughput. Molecular Devices introduces Fura-2 QBT™ Calcium Kit, a homogeneous ratiometric calcium flux assay that incorporates our signature QBT quench based technology with Fura-2 dye. The advantages of this kit are the removal of the wash step in the assay protocol which saves time and buffer costs. It offers less well to well variability, and a larger signal window. Here, we compare calcium flux responses from the new kit to the traditional Fura-2 wash assay and a competitor kit. Whether used with a FlexStation® 3 Microplate Reader or with the FLIPR® Tetra System fitted with UV excitation LEDs (340 and 380 nm) and a 475-535 nm emission filter, the new Fura-2 QBT Calcium Kit enables homogeneous, ratiometric calcium flux assays for both research and drug discovery applications.

Assay Principle

Fura-2 AM is a calcium sensitive indicator that is excited at 340nm and 380 nm, with emission at 510 nm. The dye undergoes an absorption shift from 380 nm to 340 nm when bound to calcium ions. Figure 1 shows a GPCR mediated calcium flux within a cell causing the Fura-2 QBT Dye 340/510nm emission signal to increase while the 380/510 nm emission signal decreases. The ratio of the emission signals is calculated to measure the maximum – minimum signal response for the concentration response curves.

Figure 1. Change in Fura-2 QBT Kit dye signal during calcium flux

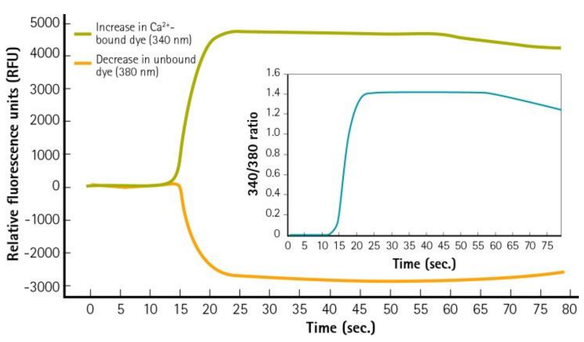


Figure 1. In this diagram, raw signal changes in response to changes in intracellular calcium concentration are shown in orange and green. Both signals are measured at 510 nm emission. Increase in calcium bound to Fura-2 causes the dye emission signal from 340 nm excitation to increase and the dye emission signal from 380 nm excitation to decrease. Shown in the inset, 340/380 nm ratio at 510 nm emission is used calculate the concentration response curve.

Materials and Methods

Fura-2 QBT Calcium Kit, Explorer format (PN #R8197) was used in this study. Dye was resuspended in Hank's Balanced Salt Solution (HBSS) with 20 mM HEPES. 2.5 mM probenecid (PBX) was added where necessary to inhibit dye leaking out of cells. Adherent CHO M1, HEK-293, or HeLa cells (all from ATCC) were plated the night before the assay in 384-well black and clear cell plates and incubated at 37° 5% CO_2. The microplates were removed from the incubator the next day and 25 µL Fura-2 QBT dye or competitor kit dye loading buffer was added to wells containing 25 µL assay buffer or media. Plates loaded with the Fura-2 QBT Kit (PN #R8197) or BD Ratiometric Calcium Kit (#644243) were incubated one hour at 37°C 5% CO_2. A traditional Fura-2 wash protocol method was also used for comparison studies. Cells were incubated with 4 µM Fura-2 dye for one hour at 37°C 5% CO_2 followed by 3 washes in HBSS buffer + 20 mM HEPES using an AquaMax® 4000 Microplate Washer. Cells were allowed to rest for 15 minutes before being read on the instruments.

Compound plates, at 5X final concentration were prepared in HBSS + 20 mM HEPES. 12.5 µL. Compound was added during detection on either the FLIPR Tetra System or the FlexStation 3 Microplate Reader. using parameters optimized for a ratiometric assay. Dye excitation was carried out at 340 and 380 nm wavelengths. Signals at 510 nm were detected, then measured in Relative Fluorescence Units (RFU) for both excitation wavelengths in each well for approximately 90 seconds, including during and post addition. Output calculated was the ratio between 340 nm and 380 nm wavelength signals at each time point during the assay. From the ratiometric signal trace, maximum -minimum value was calculated. Data for graphs and EC_{50}/IC_{50} concentrations was exported from ScreenWorks® or SoftMax Pro Software, then calculated using GraphPad Prism. Z-factor calculations were performed using the method described by Zhang, *et. al.*

Results

FLIPR ScreenWorks software calculates Fura-2 ratiometric values

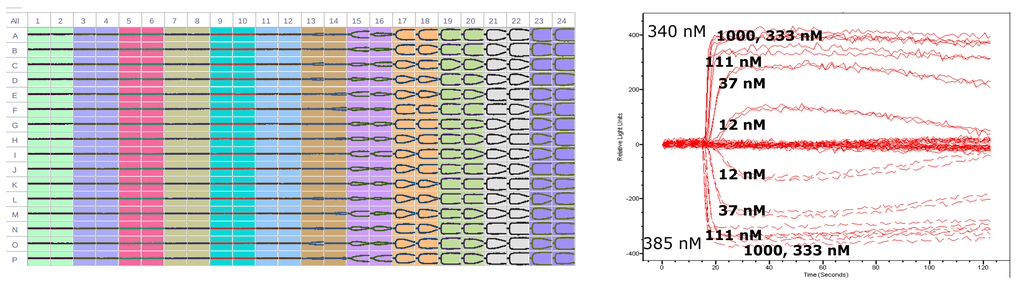
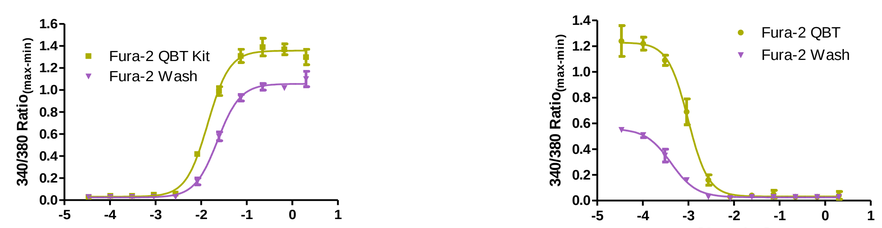


Figure 2. One the left, FLIPR ScreenWorks software screenshot showing the increase or decrease in the Fura-2 340/510 nm and 380/510 nm signal traces as the concentration of ligand increases. On the right is a detail graph from selected wells showing the signal traces at various concentrations.

Results cont.

Comparison of Fura-2 QBT Calcium Kit to Fura-2 wash protocol in CHO M1 Cells

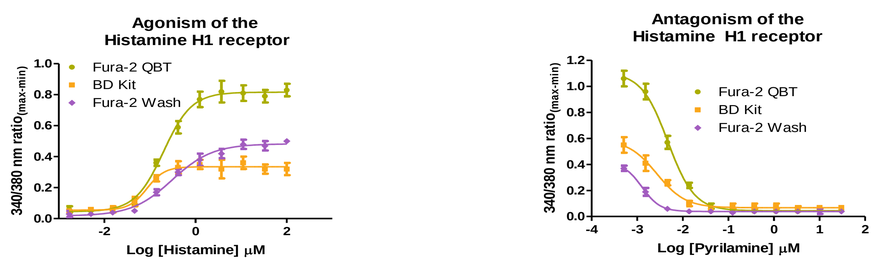


	Fura-2 QBT	Fura-2 WASH
EC_{50} (nM)	13	22
Z @EC_{80}	0.85	0.75
Window	1.3	1.03

	Fura-2 QBT	Fura-2 WASH
IC_{50} (nM)	0.98	0.4
Z IC_{80}	0.86	0.54
Window	1.2	0.53

Figure 3. CHO-M1 cells were plated in black and clear 384-well plates and incubated overnight at 37ºC 5% CO_2. On the day of the assay, 25 µL of each of the three dyes containing 2.5 mM probenecid was added to respective wells containing 25 µL culture media and the plates were returned to the incubator for one hour. The Fura-2 dye alone was washed three times on the AquaMax 4000 Microplate Washer. All plates were allowed to rest 15 minutes prior to running on the FLIPR Tetra System with UV LEDs and 475-535 nm filter. In this assay, 5 mM probenecid was added to the loading buffer to help the cells retain the dye. Fura-2 QBT Calcium Kit exhibits a larger signal window as well as higher Z factors @ EC_{80} concentration compared with the traditional Fura-2 wash assay. This is especially noticeable in the atropine antagonism assay against 50 nM carbachol challenge agonist.

Fura-2 QBT Calcium Kit has the highest signal window

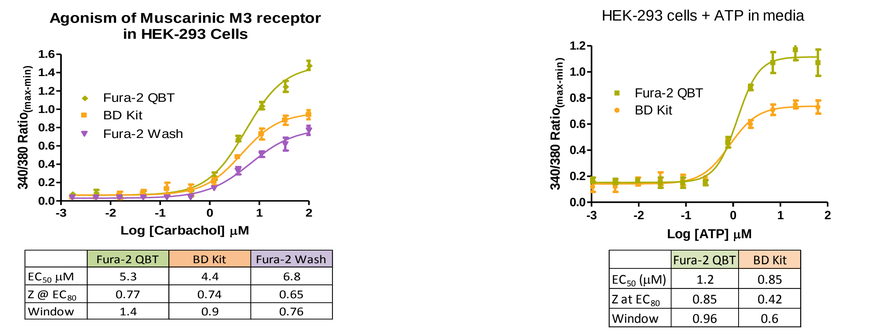


Histamine	Fura-2 QBT	BD Kit	Fura-2 Wash
EC_{50} (µM)	0.2	0.09	0.3
Z @ EC_{80}	0.72	0.53	0.69
Window	0.77	0.29	0.44

Pyrilamine	Fura-2 QBT	BD Kit	Fura-2 Wash
IC_{50} (nM)	4.7	2.7	1.3
Z @IC_{80}	0.8	0.49	0.33
Window	1.1	0.52	0.37

Figure 4. 7,500 cells per well were plated the same manner as the CHO-M1 cells in Figure 3 above. 2.5 mM probenecid was used to help keep the dye inside the cells. Agonist EC_{50} and antagonist IC_{50} values were within one-half log of each other as well as published values. In the pyrilamine assay, challenge agonist was 40 nM histamine. Fura-2 QBT Calcium kit had the largest signal window compared to both traditional wash assay and the BD kit.

HEK-293 cells : endogenous muscarinic M3 and P2Y2 receptors

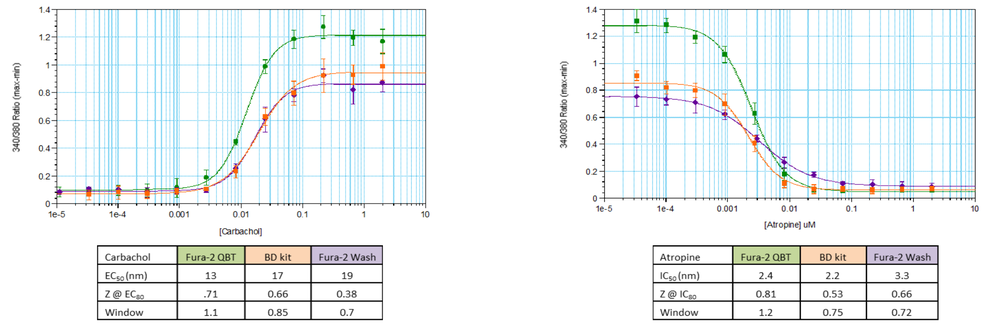


	Fura-2 QBT	BD Kit	Fura-2 Wash
EC_{50} µM	5.3	4.4	6.8
Z @ EC_{80}	0.77	0.74	0.65
Window	1.4	0.9	0.76

	Fura-2 QBT	BD Kit
EC_{50} (µM)	1.2	0.85
Z at EC_{80}	0.85	0.42
Window	0.96	0.6

Figure 5. In the figure at the left, the endogenous Muscarinic M3 receptor in HEK-293 cells is stimulated by carbachol. Challenges associated with plate washing can lead to lower Z factors due to cell blow-off as HEK-293 cells are less adherent than CHO M1 or HeLa cells. Fura-2 QBT Calcium Kit eliminates this problem. In the figure at the right, ATP stimulation of the P2Y2 receptor has a higher signal window and much higher Z factors @ EC_{80}.

Fura-2 QBT Calcium Kit provides largest signal window with CHO-M1 cells using FlexStation 3 Plate Reader



Carbachol	Fura-2 QBT	BD kit	Fura-2 Wash
EC_{50}(nm)	13	17	19
Z @ EC_{80}	.71	0.66	0.38
Window	1.1	0.85	0.7

Atropine	Fura-2 QBT	BD kit	Fura-2 Wash
IC_{50}(nm)	2.4	2.2	3.3
Z @ IC_{80}	0.81	0.53	0.66
Window	1.2	0.75	0.72

Figure 6. The FlexStation3 Instrument was used to duplicate the same assay run on the FLIPR Tetra System. Again, Fura-2 QBT Calcium Kit gave the largest signal window and the highest Z factors at EC_{80} when compared to the BD Kit and the traditional wash assay. EC_{50} values are well within one-half log and comparable to the values from the FLIPR Tetra System.

Summary

Fura-2 QBT Calcium Kit offers a homogenous alternative to the traditional Fura-2 assay by eliminating the need for washing through use of the Molecular Devices proprietary quench based technology. This improves throughput, reduces assay variability associated with uneven cell plating or loss of cells during washing, and eliminates assay costs (buffers and requirement for assay repeats due to variability). Compared to BD Ratiometric Calcium Kit and traditional Fura-2 wash assay, the Fura-2 QBT Calcium Kit provides the largest signal window and strongest Z-factors at EC_{80} and IC_{80}. Because the Fura-2 dye excites in the UV range, the kit avoids interference from compounds that auto-fluoresce when excited at 488 nm. The Fura-2 QBT Calcium Kit is validated for use with the FlexStation 3 Microplate Reader and FLIPR Tetra System, providing a scalable solution from a bench top assay to a full high throughput screen.

Together through life sciences.

Molecular Devices