molecular probes®
by life technologies™

Calcium Calibration Buffer Kits

Catalog numbers C3008MP, F6774

Table 1 Contents and storage

Calcium Calibration Buffer Kit #1 *zero and 10 mM CaEGTA (2 × 50 mL)* (Cat. no. C3008MP)			
Material	**Amount**	**Concentration‡**	**Storage†**
Zero Free Calcium Buffer (Component A)	50 mL	10 mM EGTA in 100 mM KCl, 30 mM MOPS, pH 7.2	• 2°C–8°C • DO NOT FREEZE
39 µM Free Calcium Buffer (Component F)		10 mM CaEGTA in 100 mM KCl, 30 mM MOPS, pH 7.2	
Fura-2 Calcium Imaging Calibration Kit *zero to 10 mM CaEGTA, 50 µM fura-2 (11 × 1 mL)* (Cat. no. F6774)			
Material	**Amount**	**Concentration§**	**Storage†**
Zero Free Calcium Buffer (Component A)	1 mL	0 mM CaEGTA and 50 µM fura-2	• 2°C–8°C • Protect from light • DO NOT FREEZE
0.017 µM Free Calcium Buffer (Component B)		1.0 mM CaEGTA and 50 µM fura-2	
0.038 µM Free Calcium Buffer (Component C)		2.0 mM CaEGTA and 50 µM fura-2	
0.065 µM Free Calcium Buffer (Component D)		3.0 mM CaEGTA and 50 µM fura-2	
0.100 µM Free Calcium Buffer (Component E)		4.0 mM CaEGTA and 50 µM fura-2	
0.150 µM Free Calcium Buffer (Component F)		5.0 mM CaEGTA and 50 µM fura-2	
0.225 µM Free Calcium Buffer (Component G)		6.0 mM CaEGTA and 50 µM fura-2	
0.351 µM Free Calcium Buffer (Component H)		7.0 mM CaEGTA and 50 µM fura-2	
0.602 µM Free Calcium Buffer (Component I)		8.0 mM CaEGTA and 50 µM fura-2	
1.35 µM Free Calcium Buffer (Component J)		9.0 mM CaEGTA and 50 µM fura-2	
39 µM Free Calcium Buffer (Component K)		10.0 mM CaEGTA and 50 µM fura-2	
Control Buffer, no fura-2 (Component L)		10.0 mM CaEGTA (no fura-2)	• 2°C–8°C • DO NOT FREEZE

† No preservatives (e.g., sodium azide) have been added to the solutions; we recommend that the kits be used within 3 months of receipt.
‡ Prepared in 18 Mohm deionized water.
§ In 10 mM K_2EGTA, 100 mM KCl, 30 mM MOPS, pH 7.2; prepared in 18 Mohm deionized water. In addition, each vial contains 15 µm-diameter polystyrene beads in suspension at 16,000 beads per mL to serve as coverslip spacers and focusing aids.

Introduction

The Calcium Calibration Buffer Kits have been designed to aid in the determination of the dissociation constant (K_d) of fluorescent Ca^{2+} indicators at a chosen temperature, ionic strength, and pH. Using the laboratory fluorometer or quantitative imaging system, you can calculate the K_d of an ion indicator from a plot generated by scanning the excitation or emission of the indicator in the presence of 11 different Ca^{2+} concentrations. Calibration of the Ca^{2+} indicator is an essential component of calcium measurements; general reviews of the use of these indicators include those by Takahashi and others,[1] Negulescu and Machen,[2] and Kao.[3]

The **Calcium Calibration Buffer Kit #1** (Cat. no. C3008MP) employs a reciprocal dilution method, which minimizes indicator concentration errors. The kit contains 50 mL of 10 mM K_2EGTA and 50 mL of 10 mM CaEGTA. Both solutions contain 100 mM KCl and 30 mM MOPS, pH 7.2 and are prepared in deionized water (resistance ^{3}18 Mohm). You can blend these stock solutions to prepare buffers with free Ca^{2+} concentrations ranging from 0 µM to 39 µM (see Table 3, page 5).

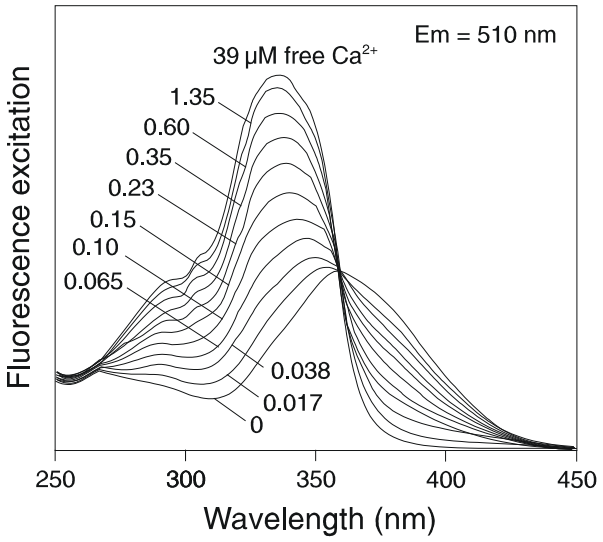
The **Fura-2 Calcium Imaging Calibration Kit** (Cat. no. F6774) is a convenient kit for fluorescence microscopy. It includes the Ca^{2+} standard solutions of the Calcium Calibration Buffer Kit #2 premixed with the fluorescent Ca^{2+} indicator, fura-2, and with polystyrene microspheres to ensure uniform coverslip/slide separation and facilitate microscope focusing. The kit contains 1 mL each of 11 pre-diluted 10 mM K_2EGTA/CaEGTA buffers containing 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 mM CaEGTA (free Ca^{2+} ranging from 0 µM to 39 µM). All the solutions contain the Ca^{2+} indicator fura-2 at 50 µM plus 100 mM KCl and 30 mM MOPS, pH 7.2. In addition, 15 µm–diameter polystyrene beads in suspension at 16,000 beads per mL have been added. The beads serve as coverslip spacers and focusing aids. A twelfth buffer, identical to the 10.0 mM CaEGTA standard but lacking fura-2, serves as a control for background fluorescence.

Principles

The buffers included in these kits are prepared according to a method described by Dr. Roger Tsien.[5] When the concentrations of Ca^{2+} and EGTA are very close to each other, the only free Ca^{2+} available is that which is in equilibrium with EGTA. The $[Ca^{2+}]_{free}$ is a function of the dissociation constant (K_d) of CaEGTA. The K_d of an indicator or chelator, defined as the concentration at which it reaches the half-saturation point, varies with ionic strength, pH, and temperature. Table 3 on page 7 shows the K_d of EGTA for Ca^{2+} in 100 mM KCl at pH values between 6.50 and 8.20 and at two different temperatures.[5] To attain a Ca^{2+} and EGTA concentration sufficiently close to each other, carefully generate a solution of the CaEGTA complex. This is accomplished by a "pH-metric" method, which makes use of the fact that the ion binding of EGTA causes an acidification of the solution. With this method, the concentrations of Ca^{2+} and EGTA can be verified to be within 0.5% of each other. In the protocol for the Calcium Calibration Buffer Kit, the highest Ca^{2+} concentration is 10 mM CaEGTA, which gives a $[Ca^{2+}]_{free}$ of about 39 µM. This $[Ca^{2+}]_{free}$ is high enough to saturate indicators with K_d values in the 0.1–1 µM range such as fura-2, indo-1, fluo-4, fluo-3, Fura Red™ and our Calcium Green™-1, Calcium Orange™, and Oregon Green® 488 BAPTA-1 indicators.

A precise set of fluorescence curves can be generated by varying the $[Ca^{2+}]_{free}$ in the solution while holding the indicator concentration, pH, ionic strength and temperature constant. The most accurate way to accomplish this is with the Calcium Calibration Buffer Kit #1. Briefly, the protocol for this kit describes the preparation of 2 dilute samples of indicator, 1 sample in 10 mM K_2EGTA ("zero Ca^{2+} sample") and the other in 10 mM CaEGTA ("high Ca^{2+} sample"). These 2 samples are then cross-diluted to produce a series of 11 solutions with the amount of total Ca^{2+} increasing by 1 mM CaEGTA with each dilution. The $[Ca^{2+}]_{free}$ in each dilution can be calculated from the K_d of CaEGTA. Although this method requires approximately 1 hour, it gives a clean isosbestic or "cross-over" point with fura-2 or indo-1 (Figure 1, page 3).

Figure 1 Spectral response of fura-2 in 0–10 mM CaEGTA buffers using Calcium Calibration Buffer Kit #1 (Cat. no. C3008MP).



Storage and handling

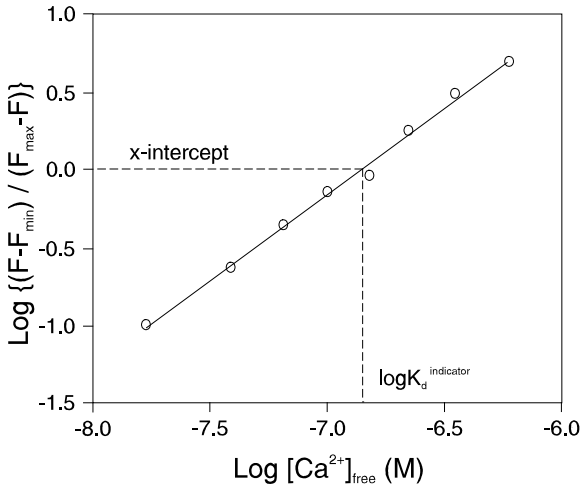
Refrigerate the buffers to retard growth of bacterial contaminants. No preservatives (e.g., sodium azide) have been added to the solutions; therefore, we recommend that the kits be used within 3 months of receipt. Do not freeze the buffers in the Fura-2 Calcium Imaging Calibration Kit, because this may damage the polystyrene microspheres.

When used according to the enclosed protocol, the Calcium Calibration Buffer Kit #1 (Cat. no. C3008MP) provides sufficient reagents for 5 complete calibrations using 2.0 mL samples in a fluorometer cuvette. When calibrating imaging equipment, there are enough kit reagents for more complete calibrations because you can use smaller volumes. The Fura-2 Calcium Imaging Calibration Kit (Cat. no. F6774) provides material sufficient for approximately 200 slides.

Specifications

Each lot of the Calcium Calibration Buffer Kits has been tested using a standard lot of fura-2 potassium salt. A representative calibration curve is shown in Figure 2. This response curve will vary with experimental conditions and instrumentation. To avoid artifacts, we have used the highest purity reagents available; the EGTA, MOPS, KCl and $MgCl_2$ have a stated purity greater than 99%.

Figure 2 Calibration curve of fura-2 with Calcium Calibration Buffer Kit #1. As a double log plot, the Ca^{2+} response of the indicator is linear with the x-intercept being equal to the log of the apparent $K_d^{Indicator}$ (145 nM from this data).



Protocol for preparing reciprocal dilutions

1.1 Prepare a stock solution of the Ca^{2+} indicator (salt form) in any dilute Ca^{2+}- and EGTA-free buffer at approximately 100–500 times the concentration required for the measurements (typically 0.2–1 mM).

1.2 Add a small aliquot of the stock indicator solution to 2 mL of Zero Free Calcium Buffer (Component A, 10 mM K_2EGTA) to give an indicator concentration of about 1–10 µM. Note that although any sample volume can be used, this example uses a 2 mL sample volume. This is the "zero Ca^{2+} sample."

1.3 Because a greater total volume of the high Ca^{2+} buffer is required for the complete series of dilutions, prepare a "high Ca^{2+} sample" by diluting exactly 3 times as much dye into 6 mL of 39 µM Free Calcium Buffer (Component B, 10 mM CaEGTA).

1.4 Verify that the pH of the 2 solutions are identical and record the pH to the nearest 0.01 units.

1.5 For measurements using a fluorometer, add exactly 2 mL of the "zero Ca^{2+} sample" in a cuvette and record the appropriate spectrum. The wavelengths used should be as shown in Table 2, but these may vary with the instrument.

Table 2 Spectral parameters for use with fluorescent Ca^{2+} indicators

Calcium Indicator	Excitation Wavelength	Emission Wavelength	Proper Spectrum
fura-2	scan 300–450 nm	490–520 nm	excitation
indo-1	340–360 nm	scan >360 nm	emission
quin-2	scan >300 nm	480–500 nm	excitation
fluo-3	480–500 nm	scan >500 nm	emission
fluo-4	480–500 nm	scan >500 nm	emission
rhod-2	540–560 nm	scan >560 nm	emission
X-rhod-1	565–585 nm	scan >590 nm	emission
Oregon Green® 488 BAPTA	480–500 nm	scan >500 nm	emission
Calcium Green™	480–500 nm	scan >500 nm	emission
Calcium Orange™	540–560 nm	scan >550 nm	emission
Calcium Crimson™	570–590 nm	scan >590 nm	emission
Fura Red™	480–500 nm	scan >550 nm	emission

1.6 Use the initial 0 mM CaEGTA/indicator sample in the cuvette to prepare the next solution by removing 0.2 mL from the sample and replacing this with an equal aliquot (0.2 mL) of the "high Ca^{2+} sample." This brings the CaEGTA concentration to 1 mM and the $[Ca^{2+}]_{free}$ to about 0.017 µM with no change in the concentration of the dye or of the total EGTA. The equation for determining the volume to remove and replace is:

[1] Volume to remove/replace = (sample volume) × {(**b** – **a**)/(**c** – **a**)}

a = current mM CaEGTA
b = desired mM CaEGTA
c = mM CaEGTA in "high Ca^{2+} sample" (typically 10.0 mM CaEGTA)

For this first dilution from 0 mM to 1 mM CaEGTA in a 2 mL sample, the remove/replace volume is calculated using equation [1] as follows:

$$2\ \text{mL} \times \frac{1\ \text{mM} - 0\ \text{mM}}{10\ \text{mM} - 0\ \text{mM}} = 0.2\ \text{mL}$$

1.7 Scan the spectrum again, then remove another aliquot (this time 0.22 mL) and replace it with 0.22 mL of the "high Ca^{2+} sample." The solution is now 2 mM CaEGTA with $[Ca^{2+}]_{free}$ of about 0.038 µM.

1.8 Record the spectrum and prepare the indicator solutions containing 3, 4, 5, 6, 7, 8, and 9 mM CaEGTA in the same way, always starting with the solution used for the previous spectrum (Table 3). For the 10 mM CaEGTA spectrum, discard the previous measurement sample and replace it with 2 mL from the "high Ca^{2+} sample" (Figure 1, page 3). Do not illuminate the solutions longer than is required to obtain the spectra. The quality of the dilutions and measurements will be obvious for those indicators that undergo excitation shifts (fura-2) or emissions shifts (indo-1) upon Ca^{2+} binding. If accurate dilutions have been made, such indicators will display a clean isosbestic point.

Table 3 Reciprocal dilutions used to arrive at the free $[Ca^{2+}]$*

CaEGTA	$[Ca^{2+}]_{free}$	Volume to remove/replace using a 2 mL sample
0 mM	0 µM	"zero Ca^{2+} sample"
1 mM	0.017 µM	Replace 0.200 mL
2 mM	0.038 µM	Replace 0.222 mL
3 mM	0.065 µM	Replace 0.250 mL
4 mM	0.100 µM	Replace 0.286 mL
5 mM	0.150 µM	Replace 0.333 mL
6 mM	0.225 µM	Replace 0.400 mL
7 mM	0.351 µM	Replace 0.500 mL
8 mM	0.602 µM	Replace 0.667 mL
9 mM	1.35 µM	Replace 1.000 mL
10 mM	39 µM	"high Ca^{2+} sample"

* See *Calculating Free Ca^{2+} Concentrations*, next page, for information about the variation of $[Ca^{2+}]_{free}$ with pH, temperature, and ionic strength.

Calculating free Ca^{2+} concentrations

Because the $[Ca^{2+}]_{free}$ value is very small in the calibration buffers, it is necessary to calculate it for each solution. Multiply the K_d of EGTA for Ca^{2+} (at the relevant pH, ionic strength, and temperature) by the molar ratio of CaEGTA to K_2EGTA in the particular solution. For example, the first dilution brings the $[Ca^{2+}]_{free}$ from essentially zero to about 0.017 µM by removing 200 µL of 10 mM K_2EGTA and replacing it with the same volume of 10 mM CaEGTA. The $[Ca^{2+}]_{free}$ is calculated from the K_d of EGTA for Ca^{2+} using equation [2]:

[2]
$$[Ca^{2+}]_{free} = K_d^{EGTA} \times \frac{[CaEGTA]}{[K_2EGTA]}$$

The ratio of CaEGTA to K_2EGTA in the 1 mM CaEGTA solution is 1:9 or 0.11. This value is multiplied by the K_d^{EGTA} at the pH, ionic strength, and temperature at which the measurement is made (Table 4, page 7). Using the reagents provided in our kits (pH 7.2 with an ionic strength of 100 mM KCl) at 20°C, the K_d^{EGTA} is 150.5×10^{-9} M. Therefore, for the 1 mM CaEGTA solution (with 9 mM K_2EGTA also present), the $[Ca^{2+}]_{free}$ is:

$$\begin{aligned}[Ca^{2+}]_{free} &= (150.5 \times 10^{-9}\ M) \times 0.11 \\ &= 1.67 \times 10^{-8}\ M \\ &= 0.0167\ \mu M\end{aligned}$$

The values for $[Ca^{2+}]_{free}$ at 20°C in solutions with pH 7.20 and ionic strength of 100 mM KCl are tabulated in Table 3 (page 5). The Ca^{2+} affinity of EGTA, which has been used to buffer the Ca^{2+} in these solutions, is very dependent on the pH, ionic strength, and temperature of the solution. As Table 4 (page 7) indicates, a change in pH of 0.05 units can alter K_d^{EGTA} by up to 20%. If your measurement is made under conditions of pH, temperature, or ionic strength that vary substantially from those represented in Table 4 (page 7), it is essential to make corrections to get the correct value for the K_d^{EGTA} for Ca^{2+}. A review by Bers and coworkers[6] in Volume 40 of the *Methods in Cell Biology* series describes methods for performing these corrections. The impact of K_d^{EGTA} corrections on Ca^{2+} measurements using fura-2 has been described by Groden and coworkers.[7]

Plotting the data

After you have recorded the spectra, plot the excitation or emission at a single wavelength against $[Ca^{2+}]_{free}$ to give a calibration curve that you can use to determine the $[Ca^{2+}]_{free}$ of an unknown solution. Similarly, for ratioable indicators such as fura-2 or indo-1, you can plot the ratio of the absorption, excitation, or emission at two wavelengths against $[Ca^{2+}]_{free}$. Ratio measurements reduce artifacts due to differences in indicator concentration, photobleaching, and path length because these factors tend to have a similar effect on the intensities at both wavelengths and they cancel in the ratio of intensities. Calculations of $K_d^{Indicator}$ are slightly more complicated when using ratio techniques.[4]

Raw spectral data and the accompanying data analysis obtained with the Calcium Calibration Buffer Kit #1 and fura-2 are shown in Figure 1 and Figure 2, respectively (see page 3). The data is plotted as the log of the $[Ca^{2+}]_{free}$ (x-axis) versus the $\log\{(F - F_{min})/(F_{max} - F)\}$ (y-axis). This double log plot gives an x-intercept that is the log of the $K_d^{Indicator}$ expressed in moles/liter. In the example, the x-intercept is −6.84. The inverse log of this number is 145×10^{-9} M (145 nM). The slope of the plot is 1.0, which reflects the 1:1 binding of each fura-2 with a single Ca^{2+} ion. The first dilution (from "zero" Ca^{2+} to 0.017 µM) has the greatest error due to contaminating ions from glassware, reagents, etc., and may sometimes be unreliable.

Table 4 Dissociation constants of EGTA for Ca^{2+} in 0.1 M KCl*

K_d^{EGTA} (nM)		
pH	**20°C**	**37°C**
6.50	3728	2646
6.60	2354	1672
6.70	1487	1057
6.75	1182	841
6.80	940	669
6.85	747	532
6.90	594	423
6.95	472	337
7.00	376	268
7.05	299	213
7.10	238	170.0
7.15	189.1	135.4
7.20	150.5	107.9
7.25	119.8	86.0
7.30	95.4	68.6
7.35	76.0	54.7
7.40	60.5	43.7
7.45	48.2	34.9
7.50	38.5	27.9
7.60	24.5	17.88
7.70	15.61	11.49
7.80	9.99	7.42
7.90	6.41	4.82
8.00	4.13	3.15
8.10	2.68	2.08
8.20	1.75	1.39

* Data from reference 5.

The Fura-2 Calcium Imaging Calibration Kit provides 11 Ca^{2+} standard buffers premixed with 50 µM fura-2. A control buffer (lacking fura-2) is also included. Each buffer also contains a dilute suspension of 15-µm polystyrene microspheres to ensure uniform coverslip/slide separation and to facilitate microscope focusing. The kit is used to calibrate Ca^{2+} measurements based on fura-2 fluorescence in digital-imaging or photometric fluorescence microscopes. With fura-2 as a Ca^{2+} indicator, you may collect data:

- as the simple fluorescence emission at 510 nm with excitation at 340 nm or
- as a ratio of emission intensities — the emission intensity at 510 nm from 340 nm excitation divided by the emission intensity at 510 nm from 380 nm excitation (see Figure 1, page 3).

The ratiometric method (*Plotting the Data*, page 6) reduces artifacts that are especially problematic in microscopy applications.

Protocol for fluorescence microscopy

2.1 Prepare a slide with each buffer solution. Shake or vortex-mix each vial vigorously immediately before sampling.

2.2 Pipet 5 µL of each calibration solution onto clean, dry microscope slides. Cover the droplets with 18 × 18-mm coverslips. Seal the edges of the coverslips with melted paraffin or other nonfluorescent sealing material.

Note: Even high-quality microscope slides and coverslips may require special cleaning prior to use to avoid spurious background fluorescence. It may be prudent to analyze the control buffer first before proceeding with the Ca^{2+} buffers containing fura-2. High background readings may indicate light scattering in the slide preparation or fluorescence from the sealing material or immersion oil. You may add protein (e.g., bovine serum albumin) to the calibrating solutions to approximate the intracellular constituents and viscosity and to reduce slide to slide variations. Protein addition, however, affects the apparent K_d of the fura-2 for Ca^{2+}.

2.3 Acquire image or photometric data, exciting the sample at 340 nm and 380 nm and measuring emission at 510 nm. Prior to taking these measurements, use transmitted light (brightfield, phase contrast, or interference microscopy) to locate a 15-µm polystyrene microsphere in the field of view and focus up and down through the bead to find the point where the diameter appears largest. After focusing in this plane, carefully move the slide on the stage to find a view without visible beads. Use this field for the fluorescence measurements.

Note: The control buffer, the zero free Ca^{2+} buffer, and the 39 µM free Ca^{2+} buffer each contribute significantly in the calculations that follow. For these buffers, we recommend taking the average of 3 determinations. For the other buffers, single determinations at 340 nm and at 380 nm are usually adequate for generating the standard curve.

2.4 Correct each emission intensity for background fluorescence. Subtract the control buffer fluorescence value (or mean value) obtained with 340 nm excitation from each 340 nm-excitation value, and subtract the control buffer fluorescence value obtained with 380 nm excitation from each 380 nm-excitation value.

2.5 Calculate the ratios. Divide the *corrected* intensity with 340 nm excitation by the corresponding *corrected* intensity with 380 nm excitation.

Plotting the data

You can use the data collected with the Fura-2 Calcium Imaging Calibration Kit to generate a standard curve, which can then be used to convert fura-2 fluorescence measurements obtained from experimental samples into estimates of free Ca^{2+} concentration. The analysis of fluorescence data taken at a single excitation wavelength (340 nm) was described above. The analysis of data taken at 2 excitation wavelengths (340 nm and 380 nm; ratiometric analysis) is described here.

The interrelationship of the free Ca^{2+} concentration and the fluorescence emission intensity ratio is described by equation [3]:

[3]
$$[Ca^{2+}]_{free} = K_d^{EGTA} \times \frac{[R - R_{min}]}{[R_{max} - R]} \times \frac{F^{380}{}_{max}}{F^{380}{}_{min}}$$

R is the ratio of 510 nm emission intensity with excitation at 340 nm, to 510 nm emission intensity with excitation at 380 nm; R_{min} is the ratio at zero free Ca^{2+}; R_{max} is the ratio at saturating Ca^{2+} (e.g., 39 µM); $F^{380}{}_{max}$ is the fluorescence intensity with excitation at 380 nm, for zero free Ca^{2+}; and $F^{380}{}_{min}$ is the fluorescence intensity at saturating free Ca^{2+}.[4]

You can calculate the free Ca^{2+} for any experimental sample from the corresponding R value. The plot of the log of $[Ca^{2+}]_{free}$ (x-axis) versus the log of $\{[(R - R_{min})/(R_{max} - R)] \times (F^{380}{}_{max}/F^{380}{}_{min})\}$ yields a straight line, the x-intercept of which is the log of K_d. An empirical value for K_d can be determined from the fura-2–containing Ca^{2+} standards.

The inclusion of 15-µm polystyrene microspheres in the calcium calibration buffers facilitates the preparation of more uniform thin films; however, it should not be assumed that the path length of these preparations is identical. Using equation [3] requires that solutions with zero and saturating Ca^{2+} contain the same fura-2 concentration and are of equal path length (i.e., the $F^{380}{}_{max}/F^{380}{}_{min}$ term depends on the assumption that $F^{380}{}_{max}$ and $F^{380}{}_{min}$ are measurements from equivalent samples). More accurate estimation of intracellular $[Ca^{2+}]$ may be accomplished simply by comparing the background-corrected 340/380 fluorescence ratio within cells with a titration curve in which the background-corrected 340/380 fluorescence ratio (y-axis) is plotted against $[Ca^{2+}]$ (x-axis).

References

1. Physiol Rev 79, 1089 (1999); **2.** Methods Enzymol 192, 38 (1990); **3.** Methods Cell Biol 40, 155 (1994); **4.** J Biol Chem 260, 3440 (1985); **5.** Methods Enzymol 172, 230 (1989); **6.** Methods Cell Biol 40, 3 (1994); **7.** Cell Calcium 12, 279 (1991); **8.** Cell Calcium 11, 85 (1990); **9.** Cell Calcium 11, 63 (1990).

Product List Current prices may be obtained from our website or from our Customer Service Department.

Cat. no.	Product Name	Unit Size
C3008MP	Calcium Calibration Buffer Kit #1 *zero and 10 mM CaEGTA (2 x 50 mL)*	1 kit
F6774	Fura-2 Calcium Imaging Calibration *zero to 10 mM CaEGTA, 50 µM fura-2 (11 x 1 mL)*	1 kit

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