Fura and Indo Ratiometric Calcium Indicators

Quick Facts

Storage upon receipt:

- ≤–20°C
- Desiccate
- Protect from light

Introduction

Since their introduction by Molecular Probes in 1985, fura-2 and indo-1 have been cited in thousands of papers that describe their applications in a wide variety of cells. These important Ca^{2+} indicators, developed by Roger Tsien and collaborators,[1] have made a major contribution to advances in the understanding of the role of calcium in cellular regulation. The ability to make ratio measurements with fura-2, indo-1, and their derivatives is an important property of these probes. At low concentrations of the indicator, use of the 340/380 nm excitation ratio for fura-2 or the 405/485 nm emission ratio for indo-1 allows accurate measurements of the intracellular Ca^{2+} concentration. Ratioing considerably reduces the effects of uneven dye loading, leakage of dye, and photobleaching, as well as problems associated with measuring Ca^{2+} in cells of unequal thickness. Measurements of indo-1 and fura-2 fluorescence can usually be made over a period of an hour without significant loss of fluorescence resulting from either leakage or bleaching. In addition, fura-2 and indo-1 are bright enough to permit measurements at intracellular concentrations of dye unlikely to cause significant Ca^{2+} buffering or damping of Ca^{2+} transients.

Fura-2 has very limited sensitivity to Ca^{2+} concentrations above 1 µM (Figure 1A). Furthermore, rapid Ca^{2+} transients monitored by fura-2 photometry are often damped due to the slow rate of Ca^{2+} dissociation from the indicator. For these reasons, Molecular Probes offers several fura-2 derivatives with lower Ca^{2+} binding affinity (i.e., higher K_d (Ca^{2+}); Table 1). Other properties of these low-affinity fura indicators, including the Ca^{2+}-dependent fluorescence excitation shift, are virtually identical to those of fura-2 (Figure 1A; Table 1) Bis-fura-2 incorporates two fura fluorophores linked to a single BAPTA chelator and therefore exhibits approximately twice the absorptivity of fura-2 (Table 1). This property results in higher fluorescence output per indicator, allowing lower dye concentrations to be used, which in turn decreases the Ca^{2+} buffering effects of the indicator. The fluorescence response to Ca^{2+} produced by bis-fura-2 is very similar to that of fura-2 (Figure 1B).

Storage and Handling

After receipt, the salts of fura-2, indo-1, and their derivatives should be stored desiccated and protected from light at ≤–20°C. These salts may be reconstituted in aqueous buffers having pH >6 and stored at 2–6°C, protected from light. DO NOT FREEZE AQUEOUS SOLUTIONS. Aqueous solutions are best used within three months.

Table 1. Spectroscopic properties and Ca^{2+} dissociation constants for fura-2, indo-1, and their derivatives.

Indicator	Catalog Number		Zero Calcium			High Calcium			K_d (Ca^{2+}) (µM)
	Salt	AM Ester	λ_A † (nm)	ϵ_{max} ‡ ($cm^{-1}M^{-1}$)	λ_F § (nm)	λ_A † (nm)	ϵ_{max} ‡ ($cm^{-1}M^{-1}$)	λ_F § (nm)	
fura-2	F1200, F6799	F1201, F1221, F1225, F14185 *	363	28,000	512 **	335	34,000	505 ††	0.14
bis-fura-2	B6810		366	56,000	511	338	68,000	504	0.37
fura-5F	F14176	F14177	363	26,000	512	336	29,000	506	0.40
fura-4F	F14174	F14175	366	21,000	511	336	23,000	505	0.77
fura-6F	F14178	F14179	364	25,000	512	336	28,000	505	5.30
fura-FF	F14180	F14181	364	25,000	510	335	28,000	506	5.50
indo-1	I1202	I1203, I1223, I1226	346	33,000	475 **	330	33,000	401 ††	0.23
indo-5F	I23912	I23913	344	31,000	471	329	31,000	398	0.47

* High-purity FluoroPure™ grade; † absorption maximum; ‡ molar extinction coefficient; § fluorescence emission maximum; ** fluorescence quantum yield 0.23 for fura-2, 0.38 for indo-1. †† Fluorescence quantum yield 0.49 for fura-2, 0.56 for indo-1. Spectroscopic data and K_d (dissociation constant) values measured in 100 mM KCl, 10 mM MOPS, pH 7.20, 0–10 mM CaEGTA at 22°C.

Dextran conjugates should be stored desiccated and protected from light at ≤–20°C. These conjugates can be dissolved in user-specified intracellular injection buffers at 10 mM (= 100 mg/mL for a 10,000 MW dextran) for microinjection into cells.

The cell-permeant acetoxymethyl (AM) esters should be stored desiccated and protected from light at ≤–20°C. Solutions should be prepared in high-quality anhydrous DMSO. Preparations of aqueous stock solutions is not recommended due to poor solubility and instability of the AM esters in water. DMSO stocks may be aliquoted and frozen for longer storage. Alternatively, specially packaged sets of 20 × 50 µg of fura-2, AM and indo-1, AM (F1221, I1223) may be purchased that can be reconstituted as required. In addition fura-2, AM and indo-1, AM are available from Molecular Probes as 1 mM solutions in anhydrous DMSO (F1225 and I1226). The AM ester forms of fura-4F, fura-5F, fura-6F, fura-FF, and indo-5F (Table 1) are supplied in sets of 10 × 50 µg. The AM ester form of bis–fura-2 is not available.

Solutions in DMSO must be tightly sealed and stored desiccated at ≤–20°C. Dissolution of the pure AM esters in DMSO may be slow, particularly the 1 mg sizes, because of the product's tendency to adhere to the walls of the vials during packaging. Warm the products and DMSO solutions to room temperature before opening. DMSO stock solutions should be used within a few weeks; however, with appropriate care they may last several months. A 1 mg/mL AM ester stock solution has a concentration of almost exactly 1 mM (molecular weights (MW) are close to 1000; exact values are printed on the product labels). Our standard specifications for the AM ester of fura-2 require minimum 95% purity by HPLC analysis. We also offer a special FluoroPure™ grade of fura-2, AM, that is specified to have ≥98% analytical purity by HPLC, in sets of 20 × 50 µg (F14185). Fura-C_{18} can be dissolved in aqueous buffers (pH >6). Fura-FF-C_{18} is less water-soluble and should initially be dissolved in ethanol.

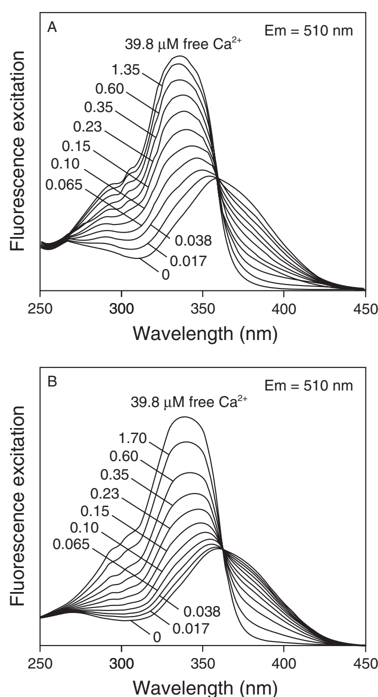

Figure 1. Fluorescence excitation spectra of A) fura-2 (F-1200) and B) bis–fura-2 (B-6810) in solutions containing zero to 39.8 µM free Ca^{2+}.

Applications

Labeling Cells with AM Esters

In general, most cells can be loaded by incubation with dilute aqueous solutions of the cell-permeant AM esters. Nonspecific esterases present in most cells hydrolyze the AM esters to formaldehyde and acetic acid, liberating the Ca^{2+}-sensitive indicator. The low leakage rate of the polyanionic indicator results in a final intracellular concentration that is much higher than the incubation concentration. The following protocol is an approximate guide to the AM ester loading conditions for fura-2, indo-1, and their derivatives:

1.1 Prepare viable cells in suspension or on a slide.

1.2 Prepare a 1–5 mM DMSO stock solution of the AM ester.

1.3 Dilute an aliquot of the DMSO stock solution into a suitable buffer. Use the minimum concentration of AM ester necessary to obtain an adequate signal; typically as low as 0.1 µM and rarely above 5 µM. Mix well. Do not store the AM esters in aqueous solution for extended periods, as spontaneous hydrolysis will occur.

1.4 The nonionic, low-toxicity detergent Pluronic® F-127 (P3000MP, P6866, P6867) may be used to facilitate AM ester solubilization if problems are encountered.

1.5 Add one volume of aqueous AM ester dispersion to one volume of cell suspension. For adherent cells rinse off the medium or buffer and replace with a solution of the AM ester. Incubate for 15 to 60 minutes at 4°C to 37°C.

1.6 Wash the cells twice with buffer and then incubate for a further 30 minutes to allow complete de-esterification of intracellular AM esters.

Cell Loading by Microinjection and Other Techniques

The following approaches are only suggestions. These methodologies should be optimized for each particular system. Fura-2 salt can be loaded into cells by pressure microinjection. Typically, injecting 1% of the cell volume with 3–30 mM dye solutions is adequate.[2,3] In experiments where electrophysiological measurements and fluorescence imaging are carried out on the same specimen, fura-2 and other indicators can be delivered intracellularly by infusion from patch pipets.[4] Typically, the indicator salt form or dextran conjugate is added to the internal pipet solution at concentrations of 50–100 µM.

We have successfully used our Influx™ pinocytic cell-loading reagent (I14402) for loading salts and dextran conjugates of ion indicators into live cells. Solutions of the salts and dextrans can also be loaded by scrape loading, bead loading, ATP-induced permeabilization, and other techniques.[5]

Measurements and Calibration for Fura Indicators

2.1 In contrast to single-wavelength indicators such as fluo-3, the absorption (or fluorescence excitation) maximum of fura indicators shifts from 363 nm for the Ca^{2+}-free chelator to about 335 nm for the Ca^{2+}-bound (Figure 1). The wavelength of maximum fluorescence emission is relatively independent of Ca^{2+}

concentration (Table 1). The largest dynamic range for Ca^{2+}-dependent fluorescence signals is obtained by using excitation at 340 nm and 380 nm and ratioing the fluorescence intensities detected at ~510 nm. From this ratio, the level of intracellular Ca^{2+} can be estimated, using dissociation constants (K_d) that are derived from calibration curves. By using the *ratio* of fluorescence intensities produced by excitation at two wavelengths, factors such as uneven dye distribution and photobleaching are minimized because they should affect both measurements to the same extent.

2.2 Once the indicator has been calibrated with solutions of known Ca^{2+} concentrations (see below), the following equation can be used to relate the intensity ratios to Ca^{2+} levels:

$$[Ca^{2+}] = K_d\, Q \frac{(R - R_{min})}{(R_{max} - R)}$$

where R represents the fluorescence intensity ratio $F_{\lambda 1}/F_{\lambda 2}$, in which λ1 (~340 nm) and λ2 (~380 nm) are the fluorescence detection wavelengths for the ion-bound and ion-free indicator, respectively. Ratios corresponding to the titration end points are denoted by the subscripts indicating the minimum and maximum Ca^{2+} concentration. Q is the ratio of F_{min} to F_{max} at λ2 (~380 nm). K_d is the Ca^{2+} dissociation constant of the indicator. Calibrating fura indicators requires making measurements for the completely ion-free and ion-saturated indicator (to determine the values for F_{min}, F_{max}, R_{min}, and R_{max}) and for the indicator in the presence of known Ca^{2+} concentrations (to determine K_d).

2.3 K_d values may be determined in a particular experimental system either by calibrating the potassium salt form of the indicator in cell-free solutions of known Ca^{2+} concentrations, or in cells that have been loaded with the AM ester form and subsequently permeabilized. Calibration solutions should closely mimic the experimental environment since the Ca^{2+} affinity of the indicator is affected by a number of variables, including pH, viscosity, temperature, protein concentration, ionic strength, and the level of other divalent cations.[6-8] Discussions of methods to correct the fura-2 dissociation constant for differences in temperature, viscosity, and ionic strength have been published.[9,10] In cell-free solutions, calibration must be done with about 1 µM indicator; higher concentrations will result in excessive absorbance, leading to significant "inner filter" effects.

2.4 Calibration solutions should be initially free of heavy metal ions such as manganese, which may affect both its fluorescence and its affinity for calcium. The divalent cation chelator TPEN (T1210) is often used to control ion levels.[11] Calcium Sponge S (C3047) is a BAPTA conjugate of water-insoluble polystyrene that can be used to selectively remove Ca^{2+} ions from buffers prior to making up solutions of known Ca^{2+} concentration. We recommend using our Fura-2 Calcium Imaging Calibration Kit (F6774) or Calcium Calibration Buffer Kits (C3008, C3009, C3721, C3722, and C3723), which contain the appropriate Ca^{2+} solutions (from 0 µM to 39.8 µM free Ca^{2+}), for calibrating fura-2, bis–fura-2, fura-4F, fura-5F, indo-1, and indo-5F. Our Calcium Calibration Buffer Kit #3 (C6775) provides eleven ready-to-use solutions containing from 1 µM to 1 mM free Ca^{2+} suitable for calibration of fura-6F and fura-FF.

2.5 Intracellular calibration may be achieved either by manipulating the Ca^{2+} levels inside cells using an ionophore or by releasing the indicator into the surrounding medium of known Ca^{2+} concentration via detergent lysis of the cells. Ca^{2+} exchangers such as the nonfluorescent 4-bromo A-23187 (B1494)[12] or ionomycin (I24222)[13-16] are typically used at concentrations of 5–50 µM to clamp intracellular calcium levels. Digitonin[17] and Triton® X-100 [18-20] have been used with cells not easily permeabilized by ionophores. Manganous ion has been used to quench external fura-2 that has leaked from cells;[21] alternatively, it can be transported into a cell using 4-bromo A-23187 to quench the intracellular chelator fluorescence. Note that for intracellular calibration, it is assumed that all the indicator is de-esterified (if loaded as an AM ester) and available for ion binding.

2.6 One can measure the ratio of fluorescence of fura indicators with excitation at 350 nm to that at 380 nm with little loss in sensitivity. This small change in filters permits use of non-quartz optics microscopes for measurement of Ca^{2+} in single cells at a significant cost savings.

Response Measurements and Calibration for Indo Indicators

Indo-1 shares most of the advantages of fura indicators except that it is somewhat more light-sensitive.[22] In contrast to fura indicators, which exhibit large changes in absorption on Ca^{2+} binding, the emission of indo-1 shifts from about 475 nm without Ca^{2+} to about 400 nm with Ca^{2+} when excited at about 350 nm (Figure 2). Equation 1 above may be used for the calibration of indo-1. For this indicator, the value of λ1 is 405 nm while λ2 is 485 nm. Indo-1 is especially useful for flow cytometry where it is easier to change the emission filters with a single excitation source (often the ultraviolet lines of the argon-ion laser in flow cytometers), and is particularly suited for multicolor fluorescence applications. Measurements with indo-5F can be carried out using the same instrument configurations as for indo-1.

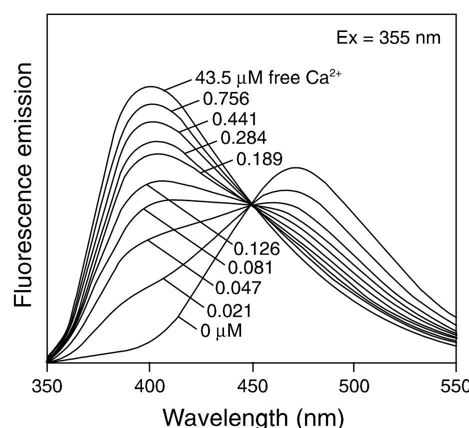

Figure 2. Fluorescence emission spectra of indo-1 (I1202) in solutions containing zero to 39.8 µM free Ca^{2+}.

Troubleshooting

Partial AM Ester Hydrolysis

Hydrolysis of all four AM esters on the chelating iminodiacetate groups is essential for a full Ca^{2+}-sensitive fluorescence response. Emission spectra of the highly fluorescent AM esters is similar to that of the Ca^{2+}-free chelator, so that failure of the cells to completely hydrolyze the AM esters will lead to a significant underestimation of the actual intracellular Ca^{2+} level. With most cells, loading requires about 30 minutes at 37°C. High AM ester concentration in the loading buffer must be avoided in order to prevent overloading intracellular esterase activity, to minimize the accumulation of the lipophilic AM esters in membrane, and to reduce formaldehyde generation from AM ester hydrolysis.

Unfortunately, some cells appear to hydrolyze the AM esters very slowly or show variable loading and therefore cannot be successfully loaded by this method. Use of excess dye as a means to maximize loading is *not* advisable because of the difficulty in removing unhydrolyzed AM esters.

Intracellular Interactions

When loading cells with the AM ester of fura-2, it has been observed that there is a tendency of the hydrolyzed indicator to compartmentalize within some cells, particularly near the nucleus. The effect is usually time dependent and may also be affected by temperature. Strong binding and fluorescence shifts induced by proteins [23] and other metals, particularly zinc, have also been reported. In some cell types, active secretion of chelator has been observed. Incomplete ester hydrolysis and compartmentalization can be avoided in cells by loading with the indicator salts or dextran conjugates available from Molecular Probes (see *Other Derivatives of Fura-2 and Indo-1*).

Indicator Leakage

Passive leakage or secretion of the indicator from cells occurs in some instances. The situation can be aggravated in cell types that actively transport the dye from the cytosol via anion pumps. It is possible to counter this to some extent by using inhibitors of the uric acid transport system such as probenecid or sulfinpyrazone.[24] The concentration of these drugs must be optimized, as excessively high concentrations will adversely affect the cells.

Autofluorescence

Autofluoresence can be a significant factor in some cells if not corrected by measurements of unloaded cells. Subtractions of the autofluorescence must be made with unlabeled cells at the same wavelengths used for calcium determinations.

Other Derivatives of Fura-2 and Indo-1

Molecular Probes' dextran conjugates of fura-2 and indo-1 tend to remain in the cytoplasm without compartmentalization or leakage. They are also less likely to bind to cellular proteins, and they can be used for long-term Ca^{2+} measurements.[25,26]

Although the spectral response curves of the conjugates are very similar to those of the free dyes, their affinity for Ca^{2+} is somewhat weaker. The dissociation constants for Ca^{2+} of fura and indo dextran in the absence of Mg^{2+} are ~350 ± 50 nM, values that can vary depending on the molecular weight of the dextran and individual batch characteristics. Dextran conjugates can be microinjected into larger cells or introduced by scrape loading, glass-beads, or other invasive techniques,[27-31] including use of our Influx™ pinocytic cell-loading reagent.

Further Resources

It is essential that the researcher be aware of the extensive published literature on the use of fura-2 and indo-1. The original paper on fura-2 and indo-1 (J Biol Chem 260, 3440 (1985)) should be consulted for methods of calibration and discussion of artifacts. Six excellent sources are:

- Cellular Calcium: A Practical Approach, J.G. McCormack and P.H. Cobbold, Eds., Oxford University Press, New York (1991), pp 1–54.
- *Calcium Signaling Protocols (Methods in Molecular Biology Volume 114)*, D. Lambert, Ed., Humana Press (1999).
- Intracellular Ion Activities and Membrane Transport in Parietal Cells Measured with Fluorescent Dyes. P.A. Negulescu and T.E. Machen, Meth Enzymol 192, 38 (1990).
- Intracellular Measurement of Calcium Using Fluorescent Probes, W.J.J.M. Scheenen, A.M. Hofer and T. Pozzan in *Cell Biology: A Laboratory Handbook, Second Edition*, Vol. 3, pp 363–374, (J.E. Celis, Ed.), Academic Press (1998).
- Measurement of Intracellular Calcium. A. Takahashi, P. Camacho, J.D. Lechleiter and B. Herman, Physiological Rev 79, 1089 (1999).

Extensive product specific bibliographies and additional information can also be obtained through our Web site (probes.invitrogen.com).

References

1. J Biol Chem 260, 3440 (1985); **2.** Biochem J 302, 5 (1993); **3.** J Microsc 166, 57 (1992); **4.** *Imaging Neurons: A Laboratory Manual*, R. Yuste, F. Lanni and A. Konnerth, Eds., Cold Spring Harbor Laboratory Press (2000) pp. 35.1–35.10; **5.** Methods Cell Biol 29, 153 (1989); **6.** Cell Calcium 11, 63 (1990); **7.** Cell Calcium 21, 233 (1997); **8.** Biochem Biophys Res Commun 177, 184 (1991); **9.** Cell Calcium 11, 85 (1990); **10.** Cell Calcium 13, 319 (1992); **11.** Biophys J 71, 1048 (1996); **12.** Cell Calcium 11, 385 (1990); **13.** Cell Calcium 21, 233 (1997); **14.** Neuron 6, 983 (1991); **15.** Pflugers Arch 418, 40 (1991); **16.** Am J Physiol 262, C128 (1992); **17.** J Biol Chem 266, 13646 (1991); **18.** Biochem Biophys Res Commun 173, 396 (1990); **19.** Biochem J 273, 541 (1991); **20.** *Cellular Calcium: A Practical Approach*, J.G. McCormack and P.H. Cobbold, Eds., Oxford University Press (1991) pp. 1–54; **21.** Cell Calcium 10, 171 (1989); **22.** Chem Biol 3, 765 (1996); **23.** Biophys J 63, 89 (1992); **24.** Cell Calcium 11, 57 (1990); **25.** J Cell Biol 58, 172 (1992); **26.** Eur J Cell Biol 58, 172 (1992); **27.** Biophys J 69, 1683 (1995); **28.** Dev Biol 166, 634 (1994); **29.** Science 217, 252 (1982); **30.** J Cell Biol 98, 1556 (1984); **31.** J Cell Sci 88, 669 (1987).

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
B1494	4-bromo A-23187, free acid	1 mg
B6810	bis-fura-2, hexapotassium salt *cell impermeant*	1 mg
C3008MP	Calcium Calibration Buffer Kit #1 *zero and 10 mM CaEGTA (2 x 50 mL)*	1 kit
C3009	Calcium Calibration Buffer Kit #2 *zero to 10 mM CaEGTA (11 x 10 mL)*	1 kit
C3047	Calcium Sponge™ S (BAPTA polystyrene)	1 g
C3721	Calcium Calibration Buffer Kit with Magnesium #1 *zero and 10 mM CaEGTA with 1 mM Mg^{2+} (2 x 50 mL)*	1 kit
C3722	Calcium Calibration Buffer Kit with Magnesium #2 *zero to 10 mM CaEGTA with 1 mM Mg^{2+} (11 x 10 mL)*	1 kit
C3723	Calcium Calibration Buffer Concentrate Kit *zero and 100 mM CaEGTA (2 x 5 mL)*	1 kit
C6775	Calcium Calibration Buffer Kit #3 *1 µM to 1 mM range (11 x 10 mL)*	1 kit
F1200	fura-2, pentapotassium salt *cell impermeant*	1 mg
F1201	fura-2, AM *cell permeant*	1 mg
F1221	fura-2, AM *cell permeant* *special packaging*	20 x 50 µg
F1225	fura-2, AM *1 mM solution in anhydrous DMSO* *cell permeant*	1 mL
F14174	fura-4F, pentapotassium salt *cell impermeant*	500 µg
F14175	fura-4F, AM *cell permeant* *special packaging*	10 x 50 µg
F14177	fura-5F, AM *cell permeant* *special packaging*	10 x 50 µg
F14178	fura-6F, pentapotassium salt *cell impermeant*	500 µg
F14179	fura-6F, AM *cell permeant* *special packaging*	10 x 50 µg
F14180	fura-FF, pentapotassium salt *cell impermeant*	500 µg
F14181	fura-FF, AM *cell permeant* *special packaging*	10 x 50 µg
F14185	fura-2, AM *FluoroPure™ grade* *special packaging*	20 x 50 µg
F3029	fura dextran, potassium salt, 10,000 MW, anionic	5 mg
F6774	Fura-2 Calcium Imaging Calibration Kit *zero to 10 mM CaEGTA, 50 µM fura-2 (11 x 1 mL)*	1 kit
F6799	fura-2, pentasodium salt *cell impermeant*	1 mg
I1202	indo-1, pentapotassium salt *cell impermeant*	1 mg
I1203	indo-1, AM *cell permeant*	1 mg
I1223	indo-1, AM *cell permeant* *special packaging*	20 x 50 µg
I1226	indo-1, AM *1 mM solution in anhydrous DMSO* *cell permeant*	1 mL
I23912	indo-5F, pentapotassium salt *cell impermeant*	500 µg
I23913	indo-5F, AM *cell permeant* *special packaging*	10 x 50 µg
I24222	ionomycin, calcium salt	1 mg
I3032	indo dextran, potassium salt, 10,000 MW, anionic	5 mg
I3033	indo dextran, potassium salt, 70,000 MW, anionic	5 mg
P3000MP	Pluronic® F-127 *20% solution in DMSO*	1 mL
P6866	Pluronic® F-127 *10% solution in water* *0.2 µm filtered*	30 mL
P6867	Pluronic® F-127 *low UV absorbance*	2 g
T1210	tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN)	100 mg

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