Revised: 01–February–2001

Influx™ Pinocytic Cell-Loading Reagent (I-14402)

Quick Facts

Storage upon receipt:
- Room temperature

Note: Keep tubes capped until immediately prior to use.

Introduction

The Influx™ pinocytic cell-loading reagent provides a convenient, rapid and simple procedure for loading water-soluble materials into live cells. With the Influx reagent, polar compounds can be introduced into many cells simultaneously without significantly altering normal cell function. The Influx reagent provides a more gentle cell-loading method than the typical cell-loading techniques of microinjection, electroporation, hypotonic shock or scrape loading, which are all physically disruptive to cells.

The Influx cell-loading technique is based on the osmotic lysis of pinocytic vesicles, a technique introduced by Okada and Rechsteiner.[1] Briefly, compounds to be loaded are mixed at high concentration with a hypertonic medium, allowing the material to be carried into the cells via pinocytic vesicles. The cells are then transferred to a hypotonic medium, which results in the release of trapped material from the pinocytic vesicles within the cells, filling the cytosol with the compound (see Figure 1). Park and colleagues showed that endosomal compartments containing the hypertonic loading medium do not fuse with lysosomes.[2] Therefore, materials introduced into cells by means of the Influx reagent are not exposed to lysosomal enzymes. Furthermore, lysosomal components are not released into the cytosol as a consequence of the procedure.

When cells are incubated with a polar fluorescent tracer without any kind of special treatment to allow entry of the probe, the cells will take up the probe via a pinocytic process. This leads to a punctate staining pattern due to compartmentalization of the tracer into endosomes (Figure 2A). In contrast, when cells are loaded with a fluorescent tracer using the Influx reagent, they demonstrate a uniform staining pattern throughout the cytosol (Figure 2B). When the mean fluorescence intensity of cells loaded by both methods is examined by flow cytometry, the intensity of the cells loaded by normal pinocytic uptake appears to be higher (Figure 2C) than that of cells loaded with the Influx reagent (Figure 2D); however, upon microscopic examination, the fluorescent compounds are compartmentalized in the former case and thus may not be in a useful form to give a true measure of the cell's cytoplasmic function.

We have shown that the Influx pinocytic cell-loading technique is highly effective for loading a diverse array of probes into a variety of cell lines. Although most cell types load quickly and easily, optimal conditions for loading must be determined for each cell type. For a partial list of cell lines and fluorescent probes that we have loaded using the Influx cell-loading reagent, please refer to Table 1.

The Influx pinocytic cell-loading reagent has been designed to load compounds into cells grown on coverslips, in suspension and in tissue culture flasks. Cell labeling can be accomplished in a single 30-minute loading cycle and may be enhanced by repetitive loading. When a large volume of probe is needed, for example when labeling cells from several culture flasks, the Influx cell-loading solution can be reused several times to help conserve materials, reduce the cost of the experiment and decrease the time spent labeling cells.

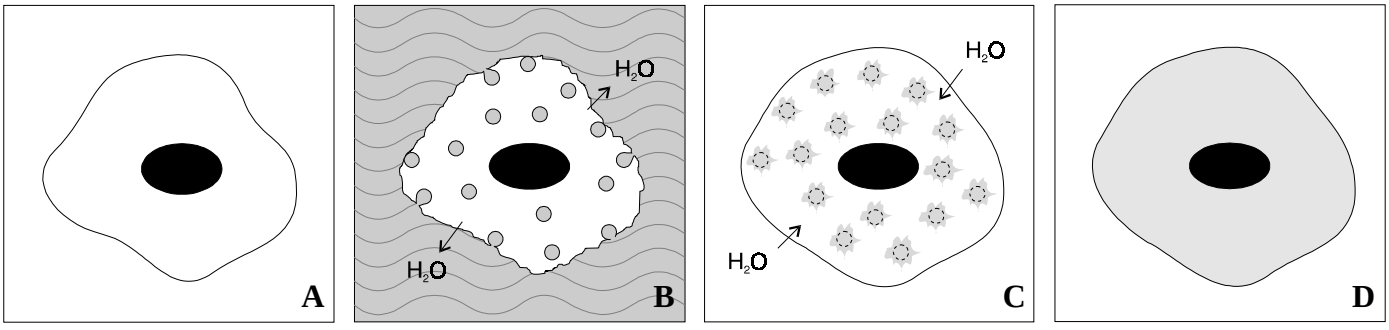

Figure 1. *Principle of the Influx reagent cell-loading method. Cultured cells (A) are exposed to Influx Hypertonic Loading Medium containing the material to be loaded, which is carried into the cells via pinocytic vesicles (B). When the cells are placed in Hypotonic Lysis Medium, the pinocytic vesicles burst (C), releasing their contents into the cytosol (D).*

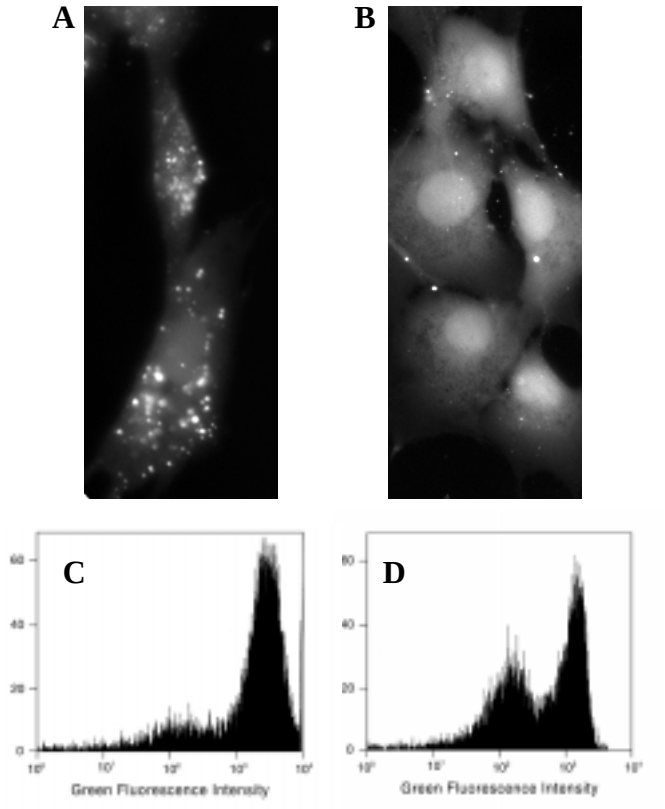

Figure 2. *CRE-BAG-2 cells loaded with Alexa Fluor® 488 hydrazide (A-10433) either by pinocytic uptake in normal growth medium, which resulted in punctate staining pattern (A), or in Influx Hypertonic Loading Medium followed by hypotonic lysis of pinosomes, which resulted in a uniform fluorescent staining pattern (B). While the mean fluorescence intensity of the punctate-stained cells, as measured by flow cytometry, was somewhat higher (C) than that of cells loaded using the Influx reagent procedure (D), the fluorescent compounds are compartmentalized in the former case, and thus not useful. Images, taken under identical conditions, were acquired with a Nikon 40X/0.75 NA Plan Fluor objective lens, fluorescein bandpass filter set and Photometrics cooled CCD camera. Flow cytometric analysis of green cell fluorescence was carried out with a Becton-Dickinson FACScan™ flow cytometer equipped with an argon-ion laser.*

Materials

Contents

Each tube of the Influx pinocytic cell-loading reagent contains an optimized mixture of sucrose crystals and polyethylene glycol (PEG), which when reconstituted makes 5 mL of Hypertonic Loading Medium, a sufficient volume to load 50 samples of cell grown on coverslips. Cells in suspension or in culture flasks may also be easily loaded; in these procedures the number of possible cell loadings varies depending on the cell suspension volume or the size of the culture flask used.

Storage and Handling

The Influx cell-loading reagent should be stored at room temperature, keeping the tubes capped until immediately prior to use. Stored properly, these materials are stable for at least two years.

Solution Preparation

Hypertonic Loading Medium

1.1 Prewarm 5 mL culture medium, without serum, to 37°C.

1.2 Melt the PEG (waxy solid on top of sucrose crystals) in one of the tubes by heating the tube in very hot water (~80°C) for at least 2 minutes.

1.3 Remove the cap and quickly add 4.7 mL of 37°C culture medium, without serum, to the tube.

1.4 Replace the cap and vortex several times over a 5 minute period to completely dissolve the sucrose crystals.

1.5 Remove the cap and add 250 µL of serum (the type required for your cell line) and 50 µL of 1 M HEPES buffer, pH 7.4, or other suitable buffer, to the tube.

1.6 Replace the cap and mix by vortexing several times.

1.7 Maintain the Hypertonic Loading Medium at the optimal temperature for your cell line. The compound to be loaded into the cells should be added to the Hypertonic Loading Medium immediately prior to use. DO NOT dilute the Hypertonic Loading Medium to less than 70% strength when adding the compound to be loaded into the cells.

Note: The Hypertonic Loading Medium may be filter-sterilized following steps 1.4 or 1.6. We recommend using a 0.8/0.2 mm Supor® Acrodisc® PF syringe filter (Gelman Sciences #4187). Filter-sterilized Hypertonic Loading Medium may be stored at 4°C for later use.

Hypotonic Lysis Medium

Prepare Hypotonic Lysis Medium by combining culture medium, without serum, and sterile deionized water in a 6:4 ratio. The volume required per loading will vary from 10–70 mL depending upon the protocol used.

Recovery Medium

Prepare 10 mL of culture medium supplemented with serum (i.e., the growth medium used for you cell line).

Experimental Protocol

The following protocols are designed for loading cells grown on coverslips, in suspension and in tissue culture flasks. Please note that each protocol is slightly different. Before beginning, please preread the protocol that you will be using and verify that you have all solutions and materials necessary. **Note:** For real-time video displays of the experimental protocols, please visit our Web site at www.probes.com.

Cell Loading Procedures for Adherent Cells on Coverslips

2.1 Prewarm at least 100 µL of Hypertonic Loading Medium containing the compound to be loaded, 10 mL of Hypotonic Lysis Medium and 10 mL of Recovery Medium, as well as all glassware, to the ideal growth temperature for your cell type. The following protocol assumes that the ideal temperature is 37°C.

2.2 Using sterile forceps, remove a coverslip from the culture dish in which the cells were grown.

2.3 Touch the edge of the coverslip to a sterile Kimwipe® to remove excess media.

2.4 Place the coverslip cell-side up in a staining dish (a 60 or 100 mm-tissue culture dish with a lid). To ensure that the coverslip does not adhere to the dish, we recommend using a "pedestal," e.g. resting the coverslip on the inverted top removed from a 1.5 mL–microfuge tube or on a 10 mm–diameter O-ring, sterilized with ethyl alcohol.

2.5 Quickly, but gently, pipet 100 µL of the prewarmed Hypertonic Loading Medium, containing the compound to be loaded, onto a *corner* of the coverslip so that the viscous Hypertonic Loading Medium will displace the small amount of residual medium without significantly diluting the loading solution.

2.6 Place the lid on the staining dish and incubate the coverslip at 37°C for 10 minutes.

2.7 Using sterile forceps, quickly, but gently, lift the coverslip and remove the excess Hypertonic Loading Medium by touching an edge of the coverslip to a sterile Kimwipe.

2.8 Place the coverslip *vertically* in a staining jar filled with *at least* 7 mL of prewarmed Hypotonic Lysis Medium, making certain that the coverslip is fully submerged. Alternatively, a coverslip mini-rack (C-14784, Figure 3) can be used conveniently to transfer the coverslip to ~30 mL of Hypotonic Lysis Medium in a standard 500 mL beaker (and later, to Recovery Medium in step 2.11).

2.9 Incubate the coverslip for *only* 2 minutes in the Hypotonic Lysis Medium. Longer exposure to the Hypotonic Lysis Medium may result in blebbing of the cell membranes and loss of cell viability.

2.10 Using sterile forceps, quickly, but gently, remove the coverslip from the Hypotonic Lysis Medium. Touch an edge of the coverslip to a sterile Kimwipe to remove excess medium.

Table 1. *The Influx reagent used for loading fluorescent probes into the cytoplasm of cultured cells.*

Compound	Cat #	Loading Concentration	Cell Lines Loaded*
Actin from rabbit muscle, Alexa Fluor 488 conjugate	A-12373	200 µg/mL	3T3, CRE-BAG-2
Alexa Fluor 488 hydrazide	A-10433	1 mg/mL	BPAE, CRE-BAG-2
Alexa Fluor 568 hydrazide	A-10434	1 mg/mL	BPAE, CRE-BAG-2
Alexa Fluor 594 hydrazide	A-10435	1 mg/mL	3T3, BPAE, CRE-BAG-2, NRK
Alexa Fluor 488 phalloidin	A-12379	300 U/mL	NRK
Calcein	C-481	1 mg/mL	3T3, A431, BPAE, CHSE, CRE-BAG-2, MDCK, J774A.1, Jurkat, NRK P3x63AG8, RAW264.7, RBL
Calcium Green-1 dextran, 3000 MW	C-6765	2 mg/mL	3T3, A431
Calcium Green-1 dextran, 70,000 MW	C-3714	2 mg/mL	CRE-BAG-2
Cascade Blue biocytin, disodium salt	C-6949	1 mg/mL	3T3, CRE-BAG-2
Fluorescein dextran, 10,000 MW	D-1820	2 mg/mL	3T3
Fluorescein dextran, anionic, lysine-fixable, 2,000,000 MW	D-7137	2 mg/mL	CRE-BAG-2
Fura dextran, 3000 MW	F-6764	5 mg/mL	A431
Fura-2, pentapotassium salt	F-1200	1 mg/mL	A431
Rhodamine Green dextran, 3000 MW	D-7163	2 mg/mL	3T3, A431, BPAE, CRE-BAG-2, RBL
Texas Red dextran, 3000 MW	D-3328	2 mg/mL	3T3, A431, BPAE, CRE-BAG-2, RBL
Texas Red dextran, 70,000 MW	D-1864	2 mg/mL	3T3, A431, BPAE, CRE-BAG-2, RBL
Tubulin from bovine brain, Oregon Green 514 conjugate	T-12391	200 µg/mL	3T3, BPAE , CRE-BAG-2
Wheat germ agglutinin, Alexa Fluor 488 conjugate	W-11261	100 µg/mL	BPAE
Wheat germ agglutinin, Texas Red-X conjugate	W-6746	100 µg/mL	BPAE

*Cell lines and their sources: BPAE, bovine pulmonary artery endothelium; MDCK, canine kidney; A431, human epidermoid carcinoma; Jurkat, human T-cell leukemia; 3T3 and CRE-BAG-2, murine fibroblasts; RAW264.7, murine monocyte-macrophage; J774A.1, murine monocyte-macrophage; P3x63AG8, murine myeloma; NRK, normal rat kidney; RBL, rat basophilic leukemia; CHSE, chinook salmon embryo.

2.11 Submerge the coverslip in 10 mL of prewarmed Recovery Medium in a new coverslip staining jar or staining dish.

2.12 Allow the cells on the coverslip to recover at 37°C for at least 10 minutes before observing in the microscope.

Loading Adherent Cells for the Attofluor® Cell Chamber

The following protocol is designed for use with the Attofluor cell chamber (A-7816), a coverslip holder designed for viewing live-cell specimens on upright or inverted microscopes (Figure 4). The cell chamber accepts 25 mm–diameter round coverslips and has an O-ring seal that prevents sample contamination by immersion oil and leakage of media from the coverslip. The chamber is also autoclavable, allowing cells to be grown directly in the chamber.

3.1 Prewarm at least 150 µL of Hypertonic Loading Medium containing the compound to be loaded, 11 mL of Hypotonic Lysis Medium and 1–5 mL of Recovery Medium, as well as an Attofluor cell chamber and all glassware, to the ideal growth temperature for your cell type. The following protocol assumes that the ideal temperature is 37°C.

3.2 Using sterile forceps, remove the 25 mm–diameter round coverslip from the culture dish in which the cells were grown.

3.3 Touch the edge of the coverslip to a sterile Kimwipe to remove excess media.

3.4 Place the coverslip cell-side up in a staining dish (a 60 or 100 mm–tissue culture dish with a lid). To ensure that the coverslip does not adhere to the dish, we recommend using a "pedestal," e.g. resting the coverslip on the inverted top removed from a 1.5 mL–microfuge tube or on a 10 mm–diameter O-ring, sterilized with ethyl alcohol.

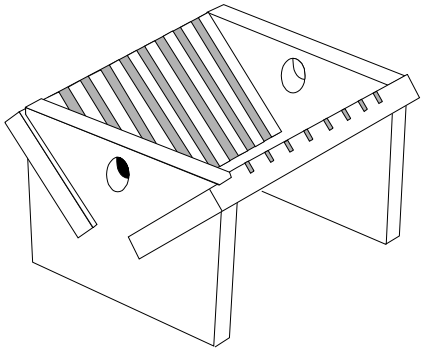

Figure 3. *Our coverslip mini-rack (C-14784) is a miniature support designed to vertically hold eight standard round or square coverslips. It fits easily into a standard 50 mL beaker and can accommodate a small stir bar beneath the rack. Use of the mini-rack eliminates the necessity for repeatedly moving coverslips between solutions with forceps. The mini-rack is handy for sequential wash steps where thorough dilution and removal of material from the coverslips is critical.*

Because it is constructed of Teflon® material, the mini-rack is low-binding, withstands strong acids and bases, is not damaged by heat and may be sterilized by a variety of methods such as autoclaving, organic solvent treatment or ethylene oxide exposure. The mini-rack is easily disassembled for cleaning and storage. Use the coverslip mini-rack during:

- *Hypotonic treatment and recovery phase of the Influx pinocytic cell-loading reagent protocol*
- *Cell permeabilization using organic solvents or detergent solutions*
- *Wash steps of immunochemical cell-staining procedures*
- *Wash steps of in situ hybridization procedures*
- *Cleaning of coverslips for calcium calibrations and critical imaging applications*
- *Surface preparation of coverslips for cell or tissue culture*

Figure 4. *The Attofluor cell chamber (A-7816) is a durable and practical coverslip holder designed for viewing live cell specimens on upright or inverted microscopes. The cell chamber is constructed from surgical stainless steel, which allows accurate temperature control when used on a heated microscope stage, and is autoclavable, allowing cells to be grown directly in the chamber. The cell chamber mounts in a standard 35 mm–diameter stage holder, while the thin 0.5 mm base thickness allows clearance for the objective when focusing. The open, beveled design facilitates microinjection or electrophysiological procedures.*

3.5 Quickly, but gently, pipet 150 µL of prewarmed Hypertonic Loading Medium, containing the compound to be loaded, onto the coverslip from the *edge* so that the viscous Hypertonic Loading Medium will slowly flow across the cell layer and displace the small amount of residual medium without significantly diluting the loading solution.

3.6 Place the lid on the staining jar and incubate the coverslip at 37°C for 10 minutes.

3.7 Using sterile forceps, quickly, but gently, lift the coverslip and remove the excess Hypertonic Loading Medium by touching the edge of the coverslip to a sterile Kimwipe.

3.8 Place the coverslip *vertically* in a staining jar filled with at least 11 mL of prewarmed Hypotonic Lysis Medium, making certain that the coverslip is fully submerged. Alternatively, a coverslip mini-rack (C-14784, Figure 3) can be used conveniently to transfer the coverslip to ~30 mL of Hypotonic Lysis Medium in a standard 50 mL beaker.

3.9 Incubate the coverslip for *only* 2 minutes in the Hypotonic Lysis Medium. Longer exposure to the Hypotonic Lysis Medium may result in blebbing of the cell membranes and loss of cell viability.

3.10 Using sterile forceps, quickly, but gently, remove the coverslip from the Hypotonic Lysis Medium, remove excess medium by touching the edge of the coverslip with a sterile Kimwipe and secure the coverslip in the prewarmed Attofluor cell chamber.

3.11 Quickly, but gently, add 1–5 mL of Recovery Medium to the coverslip in the Attofluor cell chamber by letting the solution run

down the wall of the chamber. Letting the solution run down the walls minimizes the number of cells that are dislodged.

3.12 Allow cells on the coverslip to recover at 37°C for at least 10 minutes before observing in the microscope.

Loading Cells in Suspension

4.1 Prewarm at least 20 µL of the Hypertonic Loading Medium containing the compound to be loaded, 3 mL of the Hypotonic Lysis Medium and 2 mL of the Recovery Medium, as well as all glassware, to the ideal growth temperature for your cell type. The following protocol assumes that the ideal temperature is 37°C.

4.2 Use trypsin or EDTA to remove cells from the surface of culture dishes or flasks, or use cells that are naturally in suspension.

4.3 Wash the cells to remove the trypsin or EDTA by suspending the cells in medium and then pelleting the cells by centrifugation.

4.4 Resuspend the cells in a 1 mL volume of fresh medium so that the cell density is no higher than 1×10^6 cells per mL. Transfer the cell suspension to a sterile 1.5 mL microfuge tube.

4.5 Pellet the cells by centrifugation in a microfuge for 1 minute at 2000 rpm.

4.6 Carefully remove the supernatant solution. Make sure to remove as much of the supernatant solution as possible to minimize dilution of the Hypertonic Loading Medium, which will be added next.

4.7 Add 20 µL of prewarmed Hypertonic Loading Medium containing the compound to be loaded. Gently resuspend the cells by tapping on the tube.

4.8 Incubate the cells at 37°C for 10 minutes.

4.9 Quickly, but gently, add 1 mL of the Hypotonic Lysis Medium to the cell suspension, then transfer the solution to a separate 5 mL tube containing 2 mL of Hypotonic Lysis Medium.

4.10 Aliquot the cell suspension between two 1.5 mL microfuge tubes, then incubate the cells for 1.5 minutes at 37°C. Longer exposure to the Hypotonic Lysis Medium may result in blebbing of the cell membranes and loss of cell viability.

4.11 Pellet the cells by centrifugation in a microfuge for 1 minute at 2000 rpm.

4.12 Quickly, but carefully, remove the supernatant.

4.13 Add at least 1 mL of the Recovery Medium to each microfuge tube and resuspend the cells.

4.14 Allow 10 minutes prior to observing the cells. Alternatively the cells can be immediately plated onto fresh coverslips, culture dishes or flasks for future examination.

Loading Cells in Disposable Tissue Culture Flasks

The following protocol is designed for loading large-scale cell preparations. Note that this procedure requires larger volumes of solutions than other loading protocols. Be sure to have sufficient volumes of all solutions on hand before beginning the procedure. This protocol was designed for use with 25 cm^2 Corning polystyrene tissue culture flasks (Corning #430168). Substituting flasks of different dimensions may require adaptation of this protocol. Depending on the nature and concentration of the compound to be loaded into cells, more than one flasks may be loaded sequentially using the same Hypertonic Loading Medium (Figure 5), thereby conserving the fluorescent probe and reducing the cost of your experiment.

5.1 Prewarm at least 1 mL of the Hypertonic Loading Medium containing the compound to be loaded, 70 mL of the Hypotonic Lysis Medium and 10 mL of the Recovery Medium, as well as all glassware, to the ideal growth temperature for your cell type. The following protocol assumes that the ideal temperature is 37°C.

5.2 Remove excess growth medium from the flask by decanting most of the medium and then aspirate the residual medium. It is essential that you remove as much medium as possible to minimize dilution of the Hypertonic Loading Medium, which will be added next.

5.3 Hold the flask in the upright position. Quickly, but gently, add 1 mL of the prewarmed Hypertonic Loading Medium,

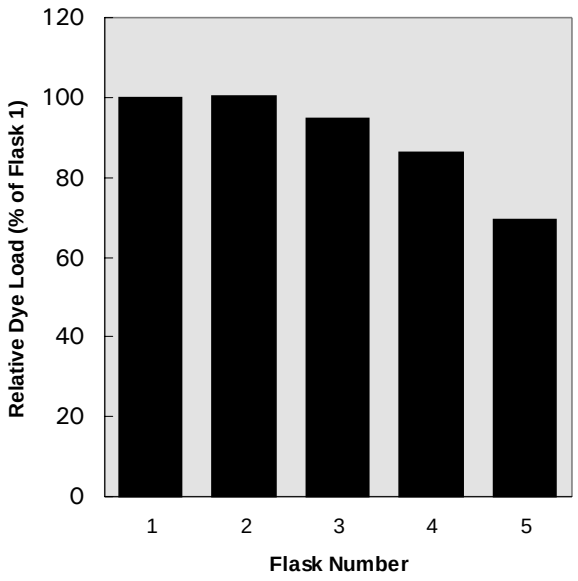

Figure 5. *Sequential loading of multiple cell preparations with one dye-containing solution of Influx pinocytic cell-loading reagent. Five different 25 cm^2 tissue culture flasks of CRE-BAG-2 fibroblasts were sequentially loaded with a single solution of the Influx reagent containing 5 mg/mL of Rhodamine Green dye–labeled 3,000 MW dextran (D-7163). The green fluorescence of cells removed from each flask was then measured by flow cytometry. The bars on the histogram represent the percent of the average cell fluorescence in a particular flask relative to the cell fluorescence achieved in the first cycle of loading.*

containing the compound to be loaded, to the bottom of the flask. Bring the flask from the upright position to a *horizontal* position with the cells on the bottom and gently rock the flask back and forth to bring the loading solution into contact with all cells.

5.4 Incubate the flask for 5 minutes at 37°C, then gently rock the flask once or twice more to redistribute any pooled medium.

5.5 Continue to incubate the flask at 37°C for an additional 5 minutes.

5.6 Bring the flask to an upright position, wait 30 seconds for the solution to run to the bottom of the flask and quickly, but gently, remove the Hypertonic Loading Medium with a pipet (e.g. with a Pasteur pipet or 1 mL pipettor). *Save this solution; it can be used to load one or more additional flasks of cells*.

5.7 Tilt the upright flask forward slightly (cells on the top). Quickly, but gently, add 70 mL of Hypotonic Lysis Medium by pouring the solution down the side of the flask away from the cells in one continuous motion. Carefully rotate the flask to the *vertical* position.

5.8 Incubate the flask *in the vertical position* for no more than 2 minutes. Longer exposure to the Hypotonic Lysis Medium may result in blebbing of the cell membranes and loss of cell viability.

5.9 Quickly, but gently, pour the Hypotonic Lysis Medium from the flask on the side away from the cells. *Note that the Hypotonic Lysis Medium should not be reused.*

5.10 Return the flask to the slightly forward position (see step 5.7). Quickly, but gently, pipet 10 mL of Recovery Medium down the side of the flask away from the cells. Rotate the flask to the horizontal position (cells on the bottom), covering the cells with medium.

5.11 Allow the cells to recover at 37°C for at least 10 minutes before use.

References

1. Cell 29, 33 (1982); **2.** J Cell Physiol 135, 443 (1988).

Product List

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

Cat #	Product Name	Unit Size
A-7816	Attofluor® cell chamber *for microscopy*	each
I-14402	Influx™ pinocytic cell-loading reagent *makes 10 x 5 mL*	1 set
C-14784	coverslip mini-rack	each

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Molecular Probes, Inc.
PO Box 22010, Eugene, OR 97402-0469
Phone: (541) 465-8300 • Fax: (541) 344-6504

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Molecular Probes Europe BV
PoortGebouw, Rijnsburgerweg 10
2333 AA Leiden, The Netherlands
Phone: +31-71-5233378 • Fax: +31-71-5233419

Customer Service: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5236850 • Fax: +31-71-5233419
eurorder@probes.nl

Technical Assistance: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5233431 • Fax: +31-71-5241883
eurotech@probes.nl