Image-iT™ FX Kits with Alexa Fluor® Secondary Detection Conjugates

Table 1. Contents and Storage Information.

Material	Amount	Concentration	Storage	Stability
Alexa Fluor® IgG conjugates	0.5 mL	2 mg/mL in 0.1 M sodium phosphate, 0.1 M NaCl, 5 mM sodium azide, pH 7.5	• ≤2–6˚C • protect from light • avoid freeze-thaw cycles	Stored undiluted as directed, product stable for at least 3 months
Alexa Fluor® streptavidin conjugates	1 mg lyophilized solid	NA	• ≤–20˚C • desiccate • protect from light	Stored as directed, product stable for at least 2 years
ProLong® Gold antifade reagent (P36930)	10 mL	1X	• ≤–20˚C • desiccate • protect from light	Stored as directed, product stable for at least 6 months
Image-iT™ FX signal enhancer (I36933) *	10 mL	1X in PBS, 2 mM sodium azide, pH 7.2	• ≤25˚C	Stored as directed, product stable for at least 6 months
CultureWell™ chambered coverglass for cell culture (C37005), sterile *	2	NA	• room temperature	Stored as directed, product stable for at least 6 months

***** Includes 2 sixteen-well chambers and a chamber removal tool.

Number of Assays: Each Image-iT™ FX Kit provides sufficient materials to perform 50–100 assays.

Approximate Fluorescence Excitation and Emission, in nm: See Table 2.

Introduction

The Image-iT™ FX Kits (Table 2) provide all of the dyes and reagents needed for optimal imaging of fixed cells and tissue sections: Alexa Fluor® goat anti–mouse IgG, goat anti–rabbit IgG, or streptavidin conjugates for superior photostability and brightness (see Table 2 for spectral characteristics of the Alexa Fluor® dyes); ProLong® Gold antifade reagent for reduced photobleaching (see *Appendix*); Image-iT™ FX signal enhancer for an improved signal-to-noise ratio; and a sample pack of two CultureWell™ chambered coverglasses for convenient processing. Each kit component is also available separately; product information sheets providing detailed information on each of these products can be found at our website (probes.invitrogen.com).

Table 2. Image-iT™ FX Kits. This table lists the catalog numbers for each Image-iT™ FX Kit and for the secondary detection reagent included in each kit.*

Fluorophore (Ex/Em †, emission color)	Goat Anti-Mouse IgG	Goat Anti-Rabbit IgG	Streptavidin
Alexa Fluor® 350 (346/442, blue)	I37150 (Kit #1) with A21049	I37155 (Kit #6) with A21068	I37160 (Kit #11) with S11249
Alexa Fluor® 488 (495/519, green)	I37151 (Kit #2) with A11029	I37156 (Kit #7) with A11034	I37161 (Kit #12) with S11223
Alexa Fluor® 555 (555/565, orange)	I37152 (Kit #3) with A21424	I37157 (Kit #8) with A21429	I37162 (Kit #13) with S21381
Alexa Fluor® 594 (590/617, red)	I37153 (Kit #4) with A11032	I37158 (Kit #9) with A11037	I37163 (Kit #14) with S11227
Alexa Fluor® 647 (650/668, near IR ‡)	I37154 (Kit #5) with A21236	I37159 (Kit #10) with A21245	I37164 (Kit #15) with S21374

* In addition, all kits include ProLong® Gold antifade reagent (10 mL, P36930), Image-iT™ FX signal enhancer (10 mL, I36933), and CultureWell™ chambered coverglasses (set of two, C37005). † Approximate fluorescence excitation (Ex) and emission (Em) maxima, in nm. ‡ Fluorescence of this long-wavelength Alexa Fluor® dye is not visible to the human eye but is readily detected by most imaging systems.

Before You Begin

Preparing Streptavidin Conjugates

1.1 Remove vial from freezer.

1.2 Add 0.5–1 mL of phosphate-buffered saline (PBS) or suitable buffer.

1.3 Ensure power has completely dissolved.

1.4 Use according to the protocol below or store undiuted at 2–6°C and protect from light. Reconstituted solutions are stable for approximately 6 months at 2–6°C with the addition of sodium azide to a final concentration of 2 mM or thimerosal to 0.2 mM. For longer storage, divide into aliquots and freeze at ≤–20°C. Avoid repeated freezing.

Using the ProLong® Gold Antifade Reagent

The ProLong® Gold antifade reagent is moisture-sensitive. Ensure that the vial is warmed to room temperature before opening. Each unit is sufficient for at least 200 coverslip-sized experiments using the protocol below.

Using the CultureWell™ Chambered Coverglass

Cells can be cultured directly in the CultureWell™ chambered coverglass, and staining procedures can be carried out in the same chambers. After removal of the chambers, the coverslip can be mounted onto a glass slide for convenience. Instructions for use are included with the chambers.

Experimental Protocol

Fixing the Cells

Below is a typical protocol for fixing cells prior to incubation with primary and secondary antibodies. This protocol should be used as a general guideline and may require further optimization for your specific application.

1.1 Rinse the cells in buffer (Hanks' Balanced Salt Solution (HBSS), PBS, or Tyrodes-HEPES) at 37°C to remove culture media. Keep the buffer warm to prevent detachment of the cells that occurs when cold buffers are added.

1.2 Fix the samples in warm (37°C) 3.7% formaldehyde (diluted in buffer); incubate 10–15 minutes at room temperature. High-quality formaldehyde is important for good results — we recommend Polysciences Cat# 18814 (16% ultrapure formaldehyde, methanol free). Slightly longer fixation times (20–30 min) may be acceptable if they do not disrupt the immunoreactivity of the target(s).

1.3 Rinse each sample in buffer 3–4 times for one minute. Cells grown on coverslips can be rinsed with buffer for 15–20 seconds.

1.4 Permeabilize the cells in 0.2% Triton X-100 (diluted in buffer) for 5 minutes or in 0.1% Triton X-100 for 15 minutes.

1.5 Rinse the samples 3–4 times in buffer.

Blocking with Image-iT™ FX Signal Enhancer

2.1 Fix and permeabilize the cells or tissue sections using either the procedure described under *Fixing the Cells* or your own procedure.

2.2 Rinse the samples with buffer.

2.3 Apply 4 drops (~200 µL) of Image-iT™ FX signal enhancer or sufficient volume to cover each coverslip or section. Incubate for 30 minutes at room temperature in a humid environment.

2.4 Rinse thoroughly with buffer.

Labeling the Primary Antibody

Please note that conditions and dilutions needed for primary antibody staining are variable from system to system. Please refer to appropriate literature or to your primary antibody provider for more specific details.

If your primary antibody is biotinylated and you are using the streptavidin conjugates for detection, it may be necessary to block endogenous biotin in your cells or tissue prior to incubation with the primary antibody, to prevent increased nonspecific binding of the streptavidin. This can be accomplished using our Endogenous Biotin Blocking Kit (E21390).

3.1 Prepare a dilution of the primary antibody in the appropriate buffer containing a suitable blocking reagent. Dilutions should be determined empirically and typically range from 1/25 to 1/1000.

Antibody labeling typically requires some type of blocking reagent included in the buffer. These blocking reagents can include one or more of the following: A) BSA (fraction V, lipid free) between 1–3% in buffer; B) 5–10% filtered goat serum or fetal calf serum — if using whole serum, centrifuge the serum and pass it through a syringe filter to remove particulate material; C) or, in some instances, Image-iT™ FX signal enhancer can be substituted for the blocking reagent; however, this may not be true for all systems.

3.2 Incubate the samples with the primary antibody. For cells, incubations are usually performed at room temperature or 37°C for 30–60 minutes. Tissue sections may require longer incubation times and temperatures of 4°C for overnight incubation, depending on the ability of the primary antibody to penetrate the tissue.

3.3 Wash cells with PBS or appropriate buffer.

Staining with Secondary Detection Conjugates

4.1. Prepare a dilution of the secondary antibody or streptavidin conjugate using PBS or another suitable buffer. Typical dilutions for secondary antibodies and streptavidin conjugates range from 1/200–1/1000. The volume needed for the incubations will depend on your setup and the surface area that needs to be covered. For the CultureWell™ chambers, a volume of 200 µL is needed for each well.

Note that because the sample has already been treated with Image-iT™ FX signal enhancer, it is not necessary to perform the 10% serum or 1–2% BSA blocking steps commonly associated with antibody staining protocols, although additional blocking steps may be performed after blocking with the Image-iT™ FX signal enhancer, if desired. Do not add serum or BSA directly to the Image-iT™ FX signal enhancer, as they can reduce the effectiveness of this product. The Image-iT™ FX signal enhancer is not displaced during subsequent wash steps.

4.2. Incubate the cells with the conjugate for 30–90 minutes at room temperature or 37°C.

4.3. Wash the sample with PBS or suitable buffer.

Mounting Samples with ProLong® Gold Antifade Reagent

The performance of ProLong® Gold antifade reagent varies depending on the dye used. ProLong® Gold antifade reagent was tested for its ability to reduce photobleaching in several dyes. The results of this experiment are found in the *Appendix*.

5.1. Remove the ProLong® Gold antifade reagent from the freezer and allow it to warm to room temperature.

5.2. Remove any excess liquid from the specimen and apply 1 drop (or suitable quantity) of ProLong® Gold antifade reagent to the specimen. Cover slide-mounted specimens with a coverslip; for specimens mounted on coverslips, place a drop of the ProLong® Gold reagent onto a clean slide and carefully lower the coverslip onto the antifade reagent to avoid trapping any air bubbles.

5.3. Allow the reagent to cure on a flat surface in the dark. Curing time may vary from a couple of hours to overnight, depending on the thickness of the sample and the relative humidity of the surrounding air. If desired, seal the coverslip to the slide after curing, in order to prevent shrinkage of the mounting medium and subsequent sample distortion. After sealing, store the slide upright in a covered slidebox at ≤–20°C. Desiccant may be added to the box to ensure that the slide remains dry.

5.4. To examine the specimen immediately after mounting, secure the coverslip at the corners using nail polish or hot wax, leaving the edges clear. After examination, the specimen can be set aside to allow the curing process to finish.

Fluorescence Microscopy Guidelines

Samples may be examined with a fluorescence microscope before the mounting medium dries. However, the antifade properties of ProLong® Gold reagent do improve slightly once it has cured. When properly stored, samples mounted in ProLong® Gold antifade reagent continue to resist photobleaching long after they are mounted.

To further reduce photobleaching, minimize the exposure of fluorescently labeled specimens to light by using neutral density filters, and expose samples only when observing or recording a signal. Optimize image capture by using a minimum of optics, high–numerical aperture objectives, relatively low magnification, high-quality optical filters, and high-speed film or high-efficiency detectors.

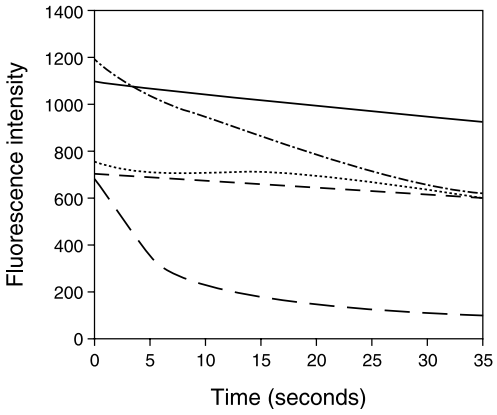
Appendix

Effectiveness of ProLong® Gold Antifade Reagent

Table 3. Resistance to photobleaching of dyes mounted in the ProLong® Gold reagent.*

Dye	30-Second Illumination	90-Second Illumination
Alexa Fluor® 488	85%	70%
Alexa Fluor® 546	67%	48%
Alexa Fluor® 555	80%	62%
Alexa Fluor® 568	93%	86%
Alexa Fluor® 594	92%	87%
Alexa Fluor® 633	121%	127%
Alexa Fluor® 635	110%	97%
Alexa Fluor® 647	100%	100%
Alexa Fluor® 660	94%	87%
Alexa Fluor® 680	96%	92%
Cy®3	80%	62%
Cy®5	100%	100%
Fluorescein	85%	65%
BODIPY® FL	54%	35%
Tetramethylrhodamine	98%	94%
Texas Red®	61%	25%
DAPI	67%	32%

*Expressed as a percent of initial fluorescence intensity remaining following either 30- or 90-second illumination, using a 40x/1.3NA objective and a 100-watt Hg-arc lamp as the light source.



ProLong® Gold antifade reagent outperforms other antifade reagents offered by Molecular Probes. BPAEC cells were labeled with fluorescein phalloidin and mounted in various antifade reagents. Samples were imaged using a 40x/1.3 NA oil immersion lens, Omega XF100-2 filter set, and frame capture rate of 1 image/second. Images were acquired using a Hamamatsu Orca ER camera using the same exposure time for all samples. Y-axis values represent averages of the highest 10% of the intensity-binned pixel values.

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

Cat #	Product Name	Unit Size
A11029	Alexa Fluor® 488 goat anti-mouse IgG (H+L) *highly cross-adsorbed* *2 mg/mL*	0.5 mL
A11032	Alexa Fluor® 594 goat anti-mouse IgG (H+L) *highly cross-adsorbed* *2 mg/mL*	0.5 mL
A11034	Alexa Fluor® 488 goat anti-rabbit IgG (H+L) *highly cross-adsorbed* *2 mg/mL*	0.5 mL
A11037	Alexa Fluor® 594 goat anti-rabbit IgG (H+L) *highly cross-adsorbed* *2 mg/mL*	0.5 mL
A21049	Alexa Fluor® 350 goat anti-mouse IgG (H+L) *highly cross-adsorbed* *2 mg/mL*	0.5 mL
A21068	Alexa Fluor® 350 goat anti-rabbit IgG (H+L) *highly cross-adsorbed* *2 mg/mL*	0.5 mL
A21236	Alexa Fluor® 647 goat anti-mouse IgG (H+L) *highly cross-adsorbed* *2 mg/mL*	0.5 mL
A21245	Alexa Fluor® 647 goat anti-rabbit IgG (H+L) *highly cross-adsorbed* *2 mg/mL*	0.5 mL
A21424	Alexa Fluor® 555 goat anti-mouse IgG (H+L) *highly cross-adsorbed* *2 mg/mL*	0.5 mL
A21429	Alexa Fluor® 555 goat anti-rabbit IgG (H+L) *highly cross-adsorbed* *2 mg/mL*	0.5 mL
C37000	CultureWell™ chambered coverglass for cell culture *sixteen wells per coverglass* *set of 8*	1 set
C37005	CultureWell™ chambered coverglass for cell culture *sixteen wells per coverglass* *sample size*	1 pack
E21390	Endogenous Biotin-Blocking Kit *100 assays*	1 kit
P36930	ProLong® Gold antifade reagent	10 mL
P36931	ProLong® Gold antifade reagent with DAPI	10 mL
S11223	streptavidin, Alexa Fluor® 488 conjugate	1 mg
S11227	streptavidin, Alexa Fluor® 594 conjugate	1 mg
S11249	streptavidin, Alexa Fluor® 350 conjugate	1 mg
S21374	streptavidin, Alexa Fluor® 647 conjugate	1 mg
S21381	streptavidin, Alexa Fluor® 555 conjugate	1 mg

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