Revised: 11–January–2001

ELF® 97 Immunohistochemistry Kit (E-6600)

Quick Facts

Storage upon receipt:

- 4°C
- Do not freeze
- Protect from light

Ex/Em of reaction product: 345/530 nm

Introduction

Molecular Probes' ELF® 97 Immunohistochemistry Kit contains our patented alkaline phosphatase substrate that yields a fluorescent precipitate — a process we call **E**nzyme-**L**abeled **F**luorescence (ELF). This soluble ELF substrate fluoresces only weakly in the blue range; however, once its phosphate is enzymatically removed, an intensely fluorescent yellow-green precipitate appears at the site of enzymatic activity.

As shown in Figure 1, the ELF precipitate exhibits a fluorescence emission that is separated from the excitation wavelength by greater than 100 nm, a characteristic that makes ELF particularly suitable for multicolor applications. For example, the ELF 97 alcohol precipitate and a blue fluorescent probe (such as Cascade Blue®–labeled secondary reagents or the counterstains DAPI and Hoechst) can be visualized simultaneously using a fluorescence microscope fitted with a standard Hoechst/DAPI long-pass filter set which is commonly supplied with fluorescence microscopes. Since the emission spectra of the ELF precipitate and these blue fluorescent labels are distinct (see Figure 1B), the two signals can be easily distinguished. In addition, as shown in Figure 1A, the excitation spectra of tetramethylrhodamine (TMR) and Texas Red® (TR) dyes are well separated from that of the ELF precipitate. Tissue that has been probed with ELF and TR- or TMR-labeled secondary reagents (or a red fluorescent counterstain such as propidium iodide) can be visualized sequentially with the appropriate filter sets without fear that bleed-through will introduce ambiguous results.

For triple-labeling, photograph your tissue through the Hoechst/DAPI long-pass filter set to record both your yellow-green ELF signal and your blue signal, and then double-expose the film through the appropriate TMR or TR filter set to record the red signal. Unlike the Hoechst/DAPI long-pass filter sets, these filter sets eliminate any blue fluorescence, yielding a blacker background.

We have successfully used the ELF substrate to characterize several antibodies against fish retina, a tissue that exhibits considerable autofluorescence when viewed with standard fluorescein and tetramethylrhodamine filter sets. Apparently, the unusually large difference between the excitation and emission

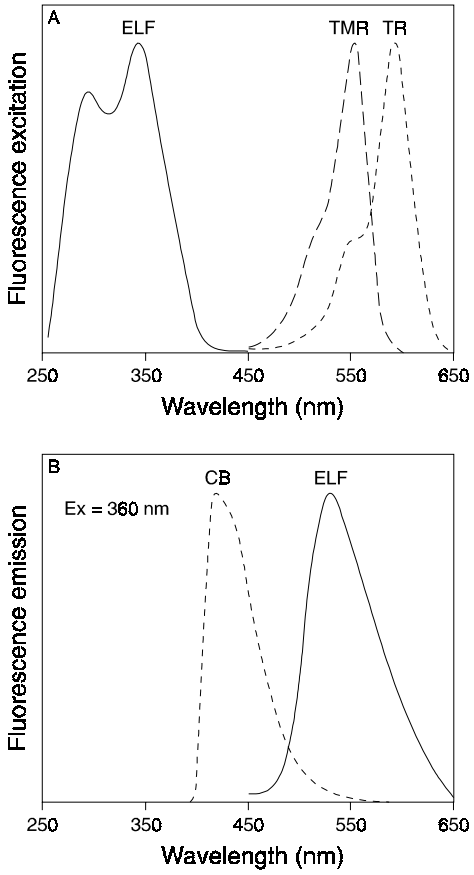

Figure 1. *A) The normalized excitation spectra of the ELF 97 alcohol precipitate (ELF), tetramethylrhodamine-labeled secondary antibody (TMR) and Texas Red–labeled secondary antibody (TR). B) The normalized emission spectra of Cascade Blue–labeled secondary antibody (CB) and ELF 97 alcohol precipitate.*

wavelengths of the ELF precipitate allows one to clearly distinguish the signal from the inherent fluorescence of the tissue. Moreover, we have shown that the ELF signal is extremely resistant to photobleaching. In a side-by-side comparison, staining with our ELF 97 phosphatase substrate was approximately 500 times more photostable than that produced by fluorescein-labeled secondary reagents.

The ELF 97 phosphatase Immunohistochemistry Kit contains our novel ELF alkaline phosphatase substrate, along with the key reagents and protocols for using this substrate to detect antigens in tissue sections. Although this ELF substrate has been used to detect EGF receptors in human carcinoma cells,[1] the techniques that were employed in these preliminary experiments yielded poor resolution and high backgrounds in immunohistochemical applications. Since the publication of this paper, we have developed reagents that greatly improve the resolution, as well as the

signal-to-noise ratio, in immunohistochemical applications. A schematic diagram of staining with the ELF 97 Immunohistochemistry Kit is shown in Figure 2. This kit provides sufficient material for staining 250–1000 tissue sections.

Materials

Reagents Supplied

- **Streptavidin** (Component A), one vial containing 5 mg of lyophilized solid
- **Alkaline phosphatase, biotin-XX conjugate** (Component B), one vial of a 250X concentrate
- **ELF 97 phosphatase substrate** (Component C), 2.5 mL of a 20X concentrate
- **Immunohistochemistry reaction buffer** (Component D), 50 mL, containing 2 mM sodium azide
- **Immunohistochemistry mounting medium** (Component E), 65 mL

Storage and Handling

Upon receipt, all kit components should be stored refrigerated at 4°C until required for use. When stored properly, these reagents are stable for approximately six months.

Materials Required but Not Provided

- Blocking buffer (30 mM Tris, 150 mM NaCl, 1% BSA, 0.5% Triton® X-100, pH 7.5)
- Wash buffer (30 mM Tris, 150 mM NaCl, 1% BSA, 0.05% Triton X-100, pH 7.5)
- Pre-reaction wash buffer (30 mM Tris, 150 mM NaCl, pH 7.5)
- Stop buffer (25 mM EDTA, 1.0 mM levamisole, 0.05% Triton X-100 in phosphate-buffered saline (PBS), pH 7.2)
- Molecular Probes offers biotinylated secondary antibodies, including goat anti-mouse (B-2763) and -rabbit (B-2770) for use in this application.

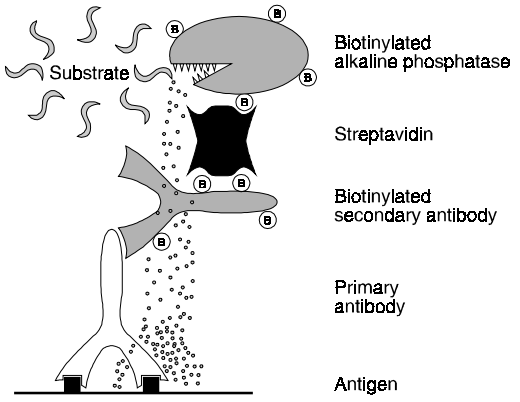

Figure 2. *Schematic diagram of the methods employed in alkaline phosphatase–mediated immunohistochemical techniques using Molecular Probes' ELF 97 Immunohistochemistry Kit. Streptavidin is used to link a biotinylated secondary antibody to the biotinylated alkaline phosphatase — a common immunohistochemical technique used to ensure that the secondary reagents adequately penetrate the tissue.*

Protocol

Reagent Preparation

1.1 The first time this protocol is carried out, the lyophilized streptavidin (Component A) should be reconstituted by adding 300 µL deionized water (dH_2O) to the product vial. To inhibit bacterial growth, sodium azide should be added to a final concentration of 2 mM (6 µL of a 100 mM sodium azide stock solution). Store the reconstituted streptavidin refrigerated at 4°C. For long-term storage, the streptavidin stock solution can be divided into single-use aliquots and stored frozen at -20°C. AVOID REPEATED FREEZING AND THAWING.

1.2 Prepare the blocking, wash and pre-reaction wash buffers according to the specifications in *Materials Required but Not Provided*. Alternatively, triethanolamine (TEA), which buffers at pH 7.5, can be substituted for Tris. These reagents should be freshly prepared and steps should be taken to minimize bacterial contamination. Any contaminating bacteria that are present when the ELF substrate is applied will become fluorescently labeled. Excess buffer may be stored for several days at 4°C.

1.3 To prepare the stop buffer, mix:

50 mL	10X PBS
25 mL	500 mM EDTA, pH 7.2 (note **A**)
5 mL	100 mM levamisole
250 µL	Triton X-100

After adjusting the pH to 7.2, bring the final volume to 500 mL with dH_2O (note **B**).

Immunohistochemistry Protocol

2.1 Prepare tissue for immunohistochemistry according to standard techniques. For example, fix the tissue in 1% or 4% formaldehyde, sink in a solution of sucrose, freeze and section. Light fixation appears to maximize the amount of antibody that binds, which may be important if the antibody is present in low concentration. However, the Triton X-100 in the diluents and washes may wash away some antigens unless the tissue is more heavily fixed.

2.2 Preincubate tissue sections for 30 minutes in blocking buffer. This presoaking step appears to be absolutely required for successful immunohistochemical staining with the ELF substrate. If 1% DMSO or 0.5% Tween® 20 is substituted, the final crystals tend to be very large.

2.3 Remove the blocking buffer. Add the primary antibody. We recommend that you prepare serial dilutions (using the wash buffer as your diluent) to determine the appropriate concentration of antibody to use (note **C**). Incubate for 30 minutes.

2.4 Wash tissue sections thoroughly with the wash buffer. We suggest cycles of three brief washes followed by a 5–10 minute incubation in wash buffer, repeated twice more.

2.5 Add 10 µg/mL of the biotinylated secondary antibody (diluted in wash buffer) to washed sections and incubate for 30 minutes.

2.6 Wash the tissue sections again as in step 2.4.

2.7 Dilute the stock streptavidin solution (prepared in step 1.1) 1:250 in the wash buffer. Prepare only the amount of diluted streptavidin solution required for the current day's experiments. Add this diluted streptavidin solution to washed sections and incubate for 30 minutes.

2.8 Wash the tissue sections again as in step 2.4.

2.9 Dilute the biotin-XX conjugate of alkaline phosphatase (Component B) 1:250 in wash buffer. Prepare only the amount of diluted enzyme conjugate solution required for the current day's experiments. Add this diluted enzyme conjugate solution to washed sections and incubate for 30 minutes.

2.10 Wash the tissue sections with the pre-reaction wash buffer (note **D**). As in step 2.4, we recommend cycles of three brief washes followed by a 5–10 minute incubation in the pre-reaction wash buffer, repeated twice more.

2.11 Dilute the ELF 97 phosphatase substrate (Component C) 20-fold in the immunohistochemistry reaction buffer (Component D). Prepare only the amount of diluted substrate solution required for the current day's experiments.

2.12 Filter the diluted substrate solutions through a 0.2 µm filter before applying it to tissue sections. We offer ELF spin filters (E-6606), which allow a very small volume to be filtered without significant loss of sample. The solution is simply added to the ELF spin filter and then centrifuged in a microcentrifuge for 10 seconds. For filtering large volumes, we recommend a syringe filter. Remove as much liquid as possible without drying tissue sections. Add filtered substrate solution to tissue sections and allow the reaction to develop. The optimal time to incubate the tissue must be determined empirically and may vary from lot to lot of enzyme conjugate. We have found a reaction time of 7–8 seconds yields excellent results in our lightly fixed cryostat sections. Others have found a longer reaction time is required for adequate signal development in formaldehyde-fixed paraffin sections (anywhere from 30 seconds to 10 minutes, depending on the antibody used). To determine the optimal reaction time, the researcher may choose to observe the reaction under a microscope equipped with the appropriate filter. If the tissue section is allowed to react for too long, an unacceptable background signal may develop. However, rinsing the tissue in buffer containing 1% BSA will remove the signal and the reaction can then be rerun.

2.13 *Immediately* stop the reaction by rapidly rinsing the tissue 12 to 15 times with the stop buffer. Incubate the slide in stop buffer for approximately 10 minutes on top of a shaker, replacing the stop buffer intermittently during the incubation. Because the substrate is apparently strongly absorbed by the tissue, this wash may be the most crucial step in the procedure. Washing is required to stop the turnover of residual substrate either by endogenous phosphatases or by nonspecifically bound biotinylated alkaline phosphatase.

2.14 Remove as much of the stop buffer as possible without drying the tissue sections.

2.15 Mount in immunohistochemistry mounting medium (Component E), which has been specially formulated to preserve the resolution of the ELF signal (note **E**). Other mounting media that we have tried cause the signal to degrade with time. Leave the mounted slides on a flat surface overnight to dry. Within a few hours, the mounting medium will have set, enabling the researcher to store the slides upright in a slide box at room temperature. With time, the mounting medium will continue to dry and may crack after a few weeks of storage. One possible solution is to seal the coverslip to the slide with melted wax after the mounting medium has partially dried (i.e., within a few days of mounting).

Visualization of ELF 97 Alcohol–Stained Tissue

ELF staining can be visualized through a standard Hoechst/DAPI long-pass filter set, which provides the appropriate UV excitation and transmits wavelengths greater than 400 nm. With this filter set, the yellow-green signal appears very distinct against a blue background. For a darker background, use a UV excitation/fluorescein emission filter set (≥515-nm long-pass or bandpass). DO NOT USE a standard fluorescein (FITC) filter set.

Notes

[A] This 500 mM EDTA stock solution can be prepared by combining equimolar amounts of NaOH and EDTA disodium salt, and then adjusting the final pH to 7.2 with a concentrated NaOH solution before bringing it to the appropriate final volume.

[B] After testing a battery of detergents, we have concluded that other detergents should not be substituted for Triton X-100 in the stop buffer. Also, the levamisole may be optional in some applications. For many applications, we have found that a pH 8 stop buffer yields both superior resolution and a better signal-to-noise ratio. However, the ELF 97 alcohol precipitate is slightly soluble at pH 8; thus, stopping the reaction with a pH 8 stop buffer may not be compatible with all applications. We recommend that researchers perform parallel experiments — using stop buffers at pH 8 (buffered with Tris) and at pH 7.2 (buffered with phosphate) — to determine the most suitable conditions for their particular experiment.

[C] We have found the required concentration of primary antibody for detection with the ELF 97 phosphate substrate is similar to that used when fluorophore-conjugated secondary reagents are employed for detection, but 10- to 20-fold greater than that used with horseradish peroxidase/diaminobenzidine methods.

[D] This washing step reduces the BSA in the tissue and yields a superior signal in our hands.

[E] This immunohistochemistry mounting medium is apparently compatible only with those nucleic stains that bind to the minor groove of the DNA helix. We have found that Hoechst 33342–stained sections remain fluorescent for several weeks when mounted in the medium included in this kit. For researchers wishing to employ other nucleic acid counterstains in their applications, tissue sections may be washed briefly with stop buffer, mounted in anhydrous glycerol and photographed immediately. The ELF signal tends to dissolve in glycerol within hours. This dissolution can be curtailed somewhat by washing extensively in stop buffer and mounting in 75% glycerol, 25% 0.15 M acetate, pH 5.

References

1. J Histochem Cytochem 41, 313 (1993).

Product List

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

Cat #	Product Name	Unit Size
E-6600	ELF® 97 Immunohistochemistry Kit	1 kit
E-6606	ELF® spin filters *20 filters*	1 box

Contact Information

Further information on Molecular Probes' products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Leiden, the Netherlands. All others should contact our Technical Assistance Department in Eugene, Oregon.

Please visit our Web site — **www.probes.com** — for the most up-to-date information

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