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ELF®97 Endogenous Phosphatase Detection Kit (E6601)

Quick Facts

Storage upon receipt:
- 4°C
- Do not freeze
- Protect from light

Ex/Em of reaction product: 345/530 nm

Introduction

Phosphatases have commonly been used as enzyme markers, allowing researchers to identify primordial germ cells,[1] to distinguish subpopulations of bone marrow stromal cells [2] and to investigate *in vitro* differentiation in carcinoma cell lines.[3-5] To facilitate such studies, Molecular Probes' scientists have developed a novel fluorescence-based method for detecting phosphatases that requires only minutes to perform. The ELF® 97 Endogenous Phosphatase Detection Kit contains our patented ELF 97 phosphatase substrate, as well as detection buffer, mounting medium and a simple protocol. This kit's soluble ELF 97 phosphatase substrate fluoresces only weakly in the blue range; however, once its phosphate is enzymatically removed, the resulting product forms an intensely fluorescent yellow-green precipitate at the site of enzymatic activity — a process we call **E**nzyme-**L**abeled **F**luorescence (ELF). Each ELF 97 Endogenous Phosphatase Detection Kit provides sufficient reagents to stain 50–200 tissue sections.

The ELF 97 alcohol precipitate exhibits a fluorescence emission that is separated from its excitation wavelength by greater than 100 nm (Figure 1). The difference between the excitation and emission wavelengths of most endogenous fluorescent components is usually much less, making the ELF 97 signal clearly distinguishable from the inherent autofluorescence of the tissue. ELF 97 staining appears yellow-green against a blue background when visualized with a Hoechst/DAPI longpass filter set, commonly supplied with fluorescence microscopes.

The ELF 97 substrate has been used to detect endogenous phosphatase activity in fixed, cultured cells [6-8] and tissue cryosections,[6,7] on bacterial colonies and biofilms [9] and in samples of marine phytoplankton.[10] Using several different tissues from zebrafish, an organism that exhibits considerable autofluorescence when viewed with standard fluorescein and tetramethylrhodamine filter sets, we compared several different methods of detecting endogenous alkaline phosphatase activity.[6,7] The ELF 97 staining was found to provide better resolution than the Gomori technique, azo dye methods and BCIP/NBT. The ELF substrate has also been successfully used in flow cytometry to analyze expression of alkaline phosphatase expression in osteogenic cell lines.[8,10]

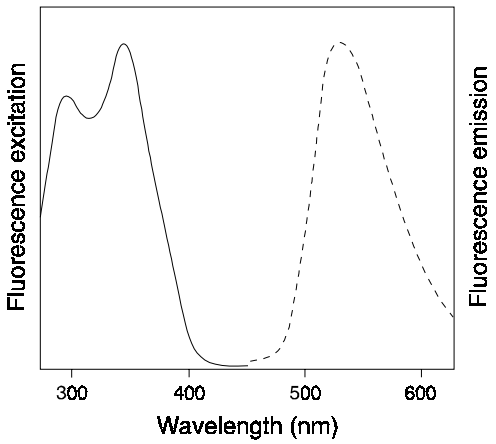

Figure 1. *The normalized excitation (–) and emission (- - -) spectra of the ELF 97 alcohol precipitate generated by the enzymatic cleavage of our soluble ELF 97 phosphatase substrate.*

Materials

Reagents Supplied
- **ELF 97 phosphatase substrate** (Component A), 500 µL of a 20 X concentrate, containing 2 mM sodium azide
- **Detection buffer** (Component B), 10 mL
- **Mounting medium** (Component C), 15 mL

Required but Not Included in Kit
- **PBS**, 25 mM EDTA, 5 mM levamisol, pH 8.0

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

Spectral Characteristics
The ELF 97 alcohol product has a maximum excitation at 345 nm and a maximum emission at 530 nm. The fluorescence can be visualized through a typical DAPI/Hoechst longpass filter set. Typical fluorescein filters will NOT work.

Protocol

Because the reaction with the phosphatase enzymes is very fast and delays may result in inferior results, we recommend that you read through the entire protocol and assemble all necessary buffers and reagents before proceeding. In addition, the microscope should be fitted with the appropriate filter sets for viewing the ELF 97 precipitate.

1. Prepare fixed cells or tissue according to standard techniques.

The quality of the ELF 97 signal is dependent on the nature of the tissue and the fixation protocol. We hypothesize that carbohydrates, lipids and protein components of cells may alter the crystallization dynamics of the ELF 97 product. The ideal conditions must be determined empirically for each sample. In general, 3.7–4% formaldehyde is a suitable fixative. The fixation time will depend greatly on the type of tissue being used and must be determined empirically. For instance, cells grown on coverslips may be fixed in 3.7% formaldehyde in phosphate buffered saline (PBS) for 10–15 minutes at room temperature and then rinsed in PBS. Acetone or ethanol are also suitable fixatives. However, methanol should be avoided because it usually will inactivate alkaline phosphatase activity.

The ELF 97 assay may be performed on cryosections. For instance, adult zebrafish tissue can be fixed in 4% formaldehyde in a phosphate buffer (100 mM phosphate, 0.15 mM $CaCl_2$, 4% sucrose, pH 7.3) for 6 hours at 4°C, rinsed in phosphate buffer and placed in 30% sucrose in phosphate buffer overnight at 4°C and then embedded, frozen and sectioned. Sections may be embedded in paraffin or material such as Tissue-Tek® OCT.

Fresh preps, such as touch preps, may potentially also be used, as long as the alkaline phosphatase is still active. It may be difficult to perform the ELF 97 detection on live cells as the detection buffer contains high salt, which may be damaging to cell morphology and cell viability. Although we have not tried the experiment, the ELF 97 signal should be stable to subsequent aldehyde fixation, although may possibly be dissolved by fixation with organic solvents such as methanol or acetone. Also, buffers having a pH above 8.0 should not be used, as the ELF 97 alcohol precipitate may dissolve.

2. Perform multiple staining with other fluorescent dyes.

If the ELF 97 assay is used in combination with other labels, such as fluorescent antibody conjugates, perform the antibody labeling first and then perform the ELF reactions. Samples stained with ELF 97 antibody may be counterstained with nuclear stains such as DAPI, Hoechst or propidium iodide *after* the ELF 97 reaction.

3. Permeabilize samples in 0.2% Tween® 20 in PBS for 10 minutes at room temperature.

While this step is optional, solubilization of cell membranes with non-ionic detergent has been shown to increase alkaline phosphatase activity.[12]

4. Rinse in PBS for at least 10 minutes.

5. Dilute the ELF 97 phosphatase substrate (Component A) 20-fold in detection buffer (Component B).

Prepare only the amount of diluted substrate solution required for the current day's experiments; 20–200 µL will be used for each sample. The concentration of the substrate given above is provided as a guideline. It is important to try a dilution series (e.g., 20-, 30-, 40-fold) to determine the optimal concentration of substrate that generates the best labeling of your sample and to not use a higher concentration of substrate than necessary. Too high of a concentration will result in overlabeling, which has a granular appearance, and possibly high background, which may appear as green fluorescent crystals all over the sample.

If too low of a concentration is used, the reaction will proceed very slowly and background crystals will appear, obscuring the signal. For first-time users, we recommend that a conventional alkaline phosphatase detection method, such as BCIP/NBT, be performed in parallel as a control for enzyme activity.

6. Filter the diluted substrate solution through a 0.2 µm pore-size filter just before applying to tissue sections.

The filtering step is required to remove any aggregates of the ELF 97 substrate that may have formed during storage. These crystals can serve as focal points for crystallization, which will lead to spurious crystals forming in the sample. We offer ELF spin filters (E6606), 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 10–30 seconds. For filtering large volumes, we recommend a syringe filter.

7. Apply the substrate solution while at the microscope.

The reaction occurs very fast, within 30–90 seconds in many cases. It is very important to perform this reaction step at the microscope so that you will be able to monitor the progress of the reaction. Before applying the substrate solution, make sure that you have the appropriate filter sets installed in your microscope. The signal can be seen through DAPI or Hoechst longpass filter or using a special filter set for ELF. If you can easily reconfigure your filter sets, you may use the excitation filter and dichroic mirror from the DAPI filter set and the emission filter from the fluorescein set. Typical fluorescein filter sets *will not work* for viewing the ELF 97 signal.

Before applying the substrate solution, wick or shake off any excess PBS from the sample, and then apply the filtered substrate solution. Generally, 20–50 µL is used for a sample on a coverslip (which will be inverted and gently placed on a slide) or 100–200 µL, to cover a tissue section mounted on a slide. Immediately, place the sample on the microscope and monitor the development of the signal, which will appear as bright yellow-green fluorescence. Reaction is usually complete within 30–90 seconds and rarely requires longer than 5 minutes. Careful monitoring of the labeling reaction is necessary to generate the best detection. The yellow-green fluorescent precipitate is very photostable and will withstand long periods of visualization. Cleavage of the ELF 97 substrate generates a very fine precipitate at the site of alkaline phosphatase activity. Large undesirable grains can form if the reaction is allowed to proceed for too long and spurious crystals may form.

Timed trials are the best way to determine how long to allow the labeling reaction to proceed. It will be helpful to stop several labeling reaction at different intervals to better visualize the progress of the signal developing. If the substrate concentration is too low and the reaction must proceed for a long time, the background signal will increase.

8. Stop the reaction by submerging the sample in wash buffer (PBS, 25 mM EDTA, 5 mM levamisol, pH 8.0).

- The pH of the wash buffer must not be above 8.0. The signal may dissolve at higher pH.
- Levamisole is included as a specific inhibitor of alkaline phosphatase activity. Alkaline phosphatases in the mammalian liver/bone/kidney family are inhibited by levamisole. In contrast, intestinal alkaline phosphatases are not generally inhibited by levamisole but can be inhibited with phenylalanine. Consult the literature for more details. (Reference 13 is a good starting point.)

9. Wash the sample with three changes of wash buffer over 10–15 minutes with gentle agitation.

It is important to wash the sample completely. Excess ELF 97 substrate left on the sample may form background crystals over a period of hours or days.

10. Remove as much wash buffer as possible without drying the sample and mount in the ELF 97 mounting medium (Component C).

If pools of liquid are left on the sample, ELF 97 alcohol crystals may develop over time. The mounting medium included in the kit has been specially formulated to preserve the resolution of the ELF 97 alcohol signal. Other mounting media may cause the signal to degrade with time. Leave mounted slides on a flat surface overnight to dry. Within a few hours, the mounting medium will have set, making it possible to store the slides upright in a slide box at room temperature.

With time, the mounting medium will continue to dry and may crack. One possible solution is to seal the coverslip to the slide with melted wax after the mounting medium has partially dried (within a few days of mounting).

11. Visualize the ELF-stained tissue.

ELF 97 staining can be visualized through a standard Hoechst/DAPI longpass 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. DO NOT USE a standard fluorescein filter because the ELF 97 precipitate will not be visible.

References

1. Anat Rec 118, 135 (1954); **2.** J Histochem Cytochem 40, 1059 (1992); **3.** Dev Biol 88, 279 (1981); **4.** Cell 5, 229 (1975); **5.** Proc Natl Acad Sci U S A 70, 3899 (1973); **6.** Pearse, A.G.E., *Histochemistry: Theoretical and Applied 3rd edition*, Baltimore, Williams & Wilkins Co. (1968); **7.** J Histochem Cytochem 47, 1443 (1999); **8.** Cytometry 37, 314 (1999); **9.** Appl Envir Microbiol 64, 1526 (1998); **10.** Mar Ecol Prog Ser 164, 21 (1998); **11.** J Histochem Cytochem 43,77 (1995); **12.** Biochim Biophys Acta 480, 403 (1977); **13.** Proc Natl Acad Sci U S A 77, 2857 (1980).

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

Cat #	Product Name	Unit Size
E6601	ELF® 97 Endogenous Phosphatase Detection Kit	1 kit
E6606	ELF® spin filters *20 filters*	1 box

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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.

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