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ELF®97 mRNA In Situ *Hybridization Kits*

E-6604 ELF® 97 mRNA *In Situ* Hybridization Kit #1
E-6605 ELF® 97 mRNA *In Situ* Hybridization Kit #2 *with streptavidin, alkaline phosphatase conjugate*

Quick Facts

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

Ex/Em of reaction product: 345/530 nm

Introduction

Molecular Probes has developed two kits for the nonisotopic detection and amplification of mRNA *in situ* hybridization signals in cells, tissue sections or whole-mount embryos. These kits employ our patented ELF® 97 phosphatase substrate,[1] which yields a brilliant, yellow-green fluorescent product at the site of enzymatic activity (Figure 1) — a process we call **E**nzyme-**L**abeled **F**luorescence (ELF). The ELF 97 precipitate is not only extremely photostable compared to commonly used fluorophores, but results in labeling that is up to 40 times brighter than signals achieved with probes directly labeled with fluorophores or with hapten-labeled probes in combination with fluorophore-labeled secondary detection reagents (Figure 2).[2,3] In our experiments, ELF alcohol precipitate–labeled samples typically require only a one-second exposure for photography, whereas fluorescein-labeled samples require at least a 45-second exposure to achieve a similar brightness. Moreover the short exposure times produce bright ELF 97 signals with negligible background fluorescence, while the long exposures of fluorescein-labeled samples often result in unacceptably high background levels.

Conventional mRNA *in situ* hybridization employs radioactively labeled DNA or RNA probes.[4-6] Signals are detected by applying a photosensitive emulsion to the microscope slide. Typically, the emulsion is exposed for days to weeks before it is developed and photographed using white-light microscopy. ELF 97 alcohol signals develop in minutes or even seconds and can be clearly distinguished from sample pigmentation, which frequently obscures both radio-active and colorimetric signals. In

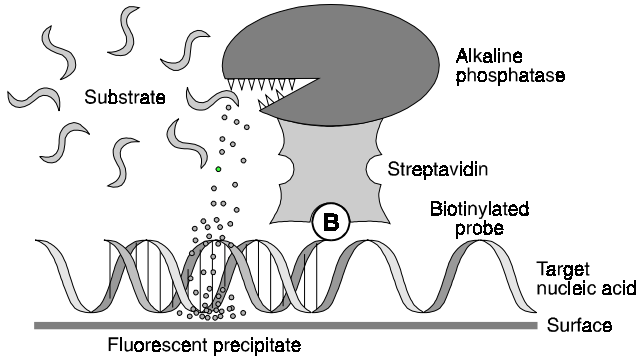

Figure 1. *Schematic diagram of the methods employed in the ELF 97 mRNA In Situ Hybridization Kits. Samples are prepared and probed with haptenylated molecules according to standard techniques. Hapten-recognizers are then applied to the sample, generally as alkaline phosphatase conjugates. Examples of hapten-recognizers that we have tested include streptavidin and antibodies directed against fluorescein, dinitrophenyl and digoxigenin. The sample is then incubated with substrate working solution. Once the ELF 97 phosphatase substrate is enzymatically cleaved, the resulting product forms an intensely fluorescent yellow-green precipitate at the site of alkaline phosphatase activity.*

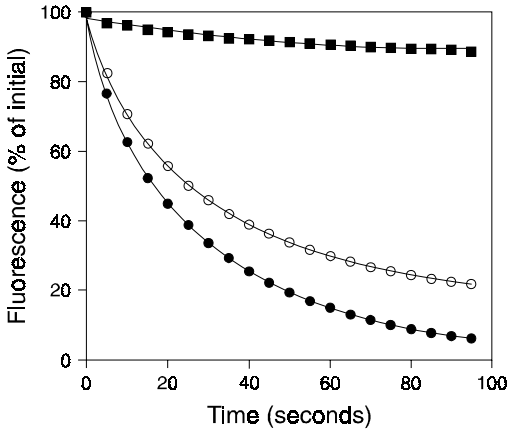

Figure 2. *Photostability comparison for ELF 97 and fluorescein-labeled tubulin preparations. Tubulin in acetone-fixed CRE BAG 2 mouse fibroblasts was labeled with an anti-tubulin monoclonal antibody (A-11126) and then detected using biotin-XX goat anti–mouse IgG (B-2763) in conjunction with either our ELF 97 Cytological Labeling Kit #2 (E-6603; ■) or fluorescein streptavidin (S-869; ●). Alternatively, anti-tubulin labeling was detected directly using fluorescein goat anti–mouse IgG (F-2761; ○). Photostablility of labeling produced by the three methods was compared by continously illuminating stained samples on a fluorescence microscope using optical signals. Images were acquired every 5 seconds using a Star 1™ CCD camera (Photometrics); the average fluorescence intensity in the field of view was calculated with Image-1® software (Universal Imaging Corp.) and expressed as a fraction of the initial intensity. Three data sets, representing different fields of view, were averaged for each conjugate to obtain the plotted time courses.*

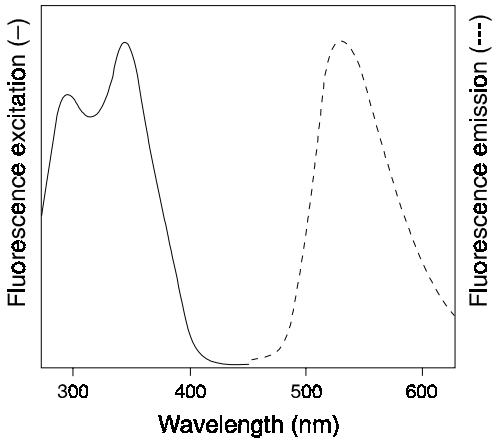

Figure 3. *The normalized excitation and emission spectra of the ELF 97 alcohol precipitate, which is generated by enzymatic cleavage of the soluble ELF 97 phosphatase substrate.*

addition, the ELF 97 precipitate is optimally excited in the ultraviolet and emits in the same spectral region as fluorescein, resulting in a Stokes shift that is greater than 100 nm (Figure 3). Thus, by employing appropriate optical filters, the ELF 97 alcohol signal can be clearly distinguished from most sample autofluorescence and from the signals of other fluorophores. This characteristic makes the ELF 97 Kits particularly suitable for multicolor applications. For example, the ELF 97 alcohol precipitate and blue fluorescent dyes — such as the DAPI and Hoechst nucleic acid counterstains — can be viewed simultaneously using a fluorescence microscope fitted with a standard Hoechst/DAPI longpass filter set. Because the ELF 97 precipitate and the blue fluorescent label have distinct emission spectra, the two signals can be easily distinguished.

The ELF 97 mRNA *In Situ* Hybridization Kits provide a complete set of reagents for using the ELF 97 phosphatase substrate to detect hybridization of biotinylated or hapten-labeled probes. Kit #2 (E-6605) provides a streptavidin–alkaline phosphatase conjugate for detecting biotinylated antibodies (B-2763, B-2770), biotinylated probes or fluoresceinated probes used in combination with biotinylated anti-fluorescein (A-982) or dinitrophenylated probes used in combination with biotinylated anti-dinitrophenyl (A-6435). Kit #1 (E-6604) is identical to Kit #2 except that the enzyme conjugate is not included. Kit #1 may be used with commercially available anti-fluorescein–alkaline phosphatase and other alkaline phosphatase conjugates. These kits provide researchers the flexibility of choosing a wide array of labeled probes for specific applications.

Materials

Reagents Supplied

- **Wash buffer** (Component A), 200 mL of a 10X concentrate
- **Blocking buffer** (Component B), 50 mL of a solution containing 2 mM sodium azide
- **Developing buffer** (Component C), 200 mL
- **ELF 97 phosphatase substrate** (Component D), 1 mL
- **Substrate additive 1** (Component E), 200 µL of a solution in DMSO
- **Substrate additive 2** (Component F), 200 µL
- **Mounting medium** (Component G), 15 mL
- **Hoechst 33342** (Component H), 100 µL of a 1 mg/mL solution
- **50 plastic coverslips** (Component I)
- **Streptavidin–alkaline phosphatase conjugate** (Component J), provided in Kit #2 (E-6605) only. This streptavidin–alkaline phosphatase conjugate has been specially formulated and tested for use in ELF applications; it is distinct from our product S-921.

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. NEVER FREEZE THE STREPTAVIDIN–ALKALINE PHOSPHATASE CONJUGATE. Substrate additive 1 (Component E), should be handled with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues. We strongly recommend using double gloves when handling the DMSO stock solution.

Reagents Required But Not Provided

- nuclease-free sterile water
- alkaline phosphatase conjugate, *for Kit #1 only*
- formaldehyde
- phosphate-buffered saline (PBS)
- bovine serum albumin (BSA)

Overview

The ELF 97 mRNA *In Situ* Hybridization Kits provide the reagents necessary to detect hybridized nucleic acid probes using the ELF 97 phosphatase substrate; however, to use Kit #1, the researcher must provide an alkaline phosphatase conjugate. Prior to ELF 97 labeling with either kit, a number of other procedures must be performed. These procedures include: making a labeled nucleic acid probe, preparing the tissue section or cells for hybridization, hybridizing the probe to the biological sample and removing unhybridized probe by appropriate washing steps. All of these steps must be optimized by the researcher prior to ELF detection. We provide protocols for preparing samples and performing hybridization. However, suggestions for prehybridization and hybridization solutions are not supplied, as these are dependent on the particular probe/target pair.

Protocols

General Considerations

The following factors should be taken into account when performing *in situ* hybridization experiments, as they affect the quality of the results. For a more detailed discussion of these parameters, see reference 7.

1.1 The type of tissue fixative as well as the fixation time must be optimized. Commonly used fixatives include formaldehyde, glutaraldehyde and ethanol/acetic acid.

1.2 Slides and coverslips should be treated with an appropriate agent to promote cell adhesion. Typical coating or "subbing" agents include gelatin, poly-L-lysine and 3-aminopropyltriethoxysilane.

1.3 The degree to which samples are deproteinated to increase the accessibility of the target to the probe should be optimized, as it is dependent on the tissue type, the fixative used, and the location (cytoplasmic or nuclear) and type (RNA or DNA) of nucleic acid targeted for hybridization. Typical methods for deproteinization include incubating samples with proteinase K or 0.2 M HCl or both.

1.4 Several characteristics of the nucleic acid probe should be optimized, including the number of haptens or biotins on the probe (i.e., the degree of substitution), the length of the linker arm between the nucleic acid backbone and the labels and the size and specificity of the probe.

1.5 The hybridization conditions should be optimized, including the time, temperature, percentage of formamide and salt concentration.

Reagent Preparation

All solutions must be maintained both nuclease-free and sterile to ensure success with the following protocols.

2.1 1X wash buffer. Dilute the 10X Wash buffer (Component A) 10-fold by adding 1800 mL sterile distilled water to the 200 mL 10X solution provided. The 1X Wash buffer may be stored at 4°C in a sterile container for up to six months.

2.2 ELF 97 phosphatase substrate working solution. Dilute the amount of ELF 97 phosphatase substrate (Component D) needed for the day's experiments 10-fold into the developing buffer (Component C). Filter the resulting solution into a sterile vial using a 0.2 µm pore-size filter, such as the ELF spin filter (E-6606), and centrifuging for 10 seconds in a microcentrifuge. These filters allow a very small volume to be filtered without significant loss of sample. For filtering large volumes, we recommend a syringe filter. After filtration, dilute the substrate additive 1 (Component E) and substrate additive 2 (Component F) each 1:500 into the substrate working solution. Vortex the solution well and use it immediately or store it at 4°C in a sterile container for up to 48 hours. For best results, prepare the substrate working solution fresh for each day's experiments. If the solution is stored for more than one day, it should be refiltered through a 0.2 µm pore-size filter to remove any precipitate before use. Note that we provide an excess of the developing buffer and substrate additives.

2.3 Post-fixation solution. Prepare a solution of 2% formaldehyde in PBS, containing 20 mg/mL bovine serum albumin. This solution can be used immediately or stored at 4°C for up to six months.

ELF Detection Protocol

This ELF detection protocol should be performed after the post-hybridization step of the in situ hybridization protocol. We provide examples below of two in situ hybridization protocols that we have found work well with ELF labeling. However, the hybridization procedure and sample preparation procedure must be optimized for each application.

3.1 After performing the post-hybridization wash steps, transfer the slide or coverslip to a jar or dish containing 1X wash buffer. Incubate the sample for 5 minutes at room temperature. *Do not let the slide or coverslip dry out at any stage of the ELF detection protocol.*

3.2 Remove the slide from the dish and place it flat in a humid chamber. Apply 100 µL of blocking reagent to the tissue section or cells and cover the slide with a plastic coverslip. Incubate the sample for 30 minutes to one hour.

3.3 While the slide is incubating,

- *for Kit #2* (E-6605), dilute the streptavidin–alkaline phosphatase conjugate (Component J) provided 1:50 into the blocking solution.
- *for Kit #1* (E-6604), prepare an appropriate dilution of an alkaline phosphatase conjugate into the blocking solution. Prepare sufficient diluted enzyme conjugate solution to allow 100 µL of solution per sample.

3.4 Remove the blocking solution from the slide. Apply 100 µL of the diluted alkaline phosphatase conjugate to the sample. Cover again with the plastic coverslip and incubate the sample for 30 minutes at room temperature. If the sample is expected to contain significant endogenous phosphatase activity, we recommend incubating samples, just prior to adding the streptavidin–alkaline phosphatase conjugate, for 1 hour in 1 mM levamisole diluted with the blocking reagent. Remove the solution and apply the streptavidin–alkaline phosphatase conjugate as described.

3.5 Wash the slide three times, for 5 minutes each time, with 1X wash buffer.

3.6 Apply 300–500 µL per slide or 100 µL per coverslip of substrate working solution to the sample. Do not cover the slide, but ensure that it does not dry out by keeping it in a humid chamber. Incubate the sample for 10 minutes to 1 hour, depending on the abundance of the signal. *Do not allow the fluorescent signal to develop for more than 2 hours because a high background consisting of large fluorescent crystals and nonspecific labeling may result.*

3.7 Remove the excess solution from the slide and view the sample. If the signal is sufficient, wash the slide immediately with 1X wash buffer. If desired, the sample can also be counterstained at this point. The blue–fluorescent nucleic acid stain provided in this kit, Hoechst 33342, may be used by diluting it in water to 1–2 µg/mL and incubating a coverslip with 100–200 µL of the staining solution or a slide with 300–500 µL of the staining solution. Samples can also be counterstained with propidium iodide; however, propidium iodide is not recommended if the sample is to be mounted with the mounting medium provided.

3.8 For long-term signal preservation, quickly wash the sample with 1X wash buffer. Post-fix the sample by incubating the slide in post-fixation solution for 15–30 minutes at room temperature. Mount the sample with mounting medium by applying 3–5 drops to each slide or 1–2 drops per coverslip. DO NOT dehydrate samples with organic solvents, such as ethanol, or mount in organic solvent–based mounting alcohol after ELF labeling. Such solvents will solubilize the ELF 97 precipitate, resulting in loss of signal.

In Situ Hybridization to mRNA in Cultured Adherent Cells

4.1 Culture cells under appropriate growth conditions on a microscope coverslip.

4.2 Wash the cells with PBS, then fix them at room temperature for 30 minutes with a solution of 4% formaldehyde and 5% acetic acid in 0.9% (w/v) NaCl.

4.3 Wash the cells with PBS at room temperature and store the coverslip in 70% ethanol at 4°C overnight, or dehydrate the cells by incubating them sequentially in 70%, 90% and 100% ethanol for 2 minutes in each solution.

4.4 To remove residual lipids, wash the cells with 100% xylene for 5–10 minutes in a glass dish. Rehydrate the cells by incubating them sequentially in 100%, 90% and 70% ethanol, followed by PBS, for 2 minutes in each solution.

4.5 To increase the accessibility of the probe to the target, treat the cells at 37°C with 0.1% (w/v) pepsin in 0.1 M HCl. *This step is unnecessary when using oligonucleotide probes.*

4.6 Wash the cells with PBS for 5 minutes at room temperature.

4.7 Post-fix the sample with 1% formaldehyde for 10 minutes. Wash the sample again with PBS.

4.8 Prehybridize the sample for 1 hour. *Prehybridization and hybridization solutions must be optimized for each probe/target pair.*

4.9 Hybridize the sample for 16 hours at 37°C. *Three hours may be sufficient for oligonucleotide probes, especially when using optimal hybridization conditions.*

4.10 Remove unbound probes by washing the sample according to standard hybridization wash procedures. *The exact procedure must be optimized for every probe/target pair. For example, for oligonucleotides we have found that three 10-minute washes with 4X SSC (0.6 M NaCl, 0.06 M sodium citrate, pH 7.0) at room temperature is usually adequate.*

4.11 At this point, other secondary detection reagents, such as biotinylated antibody conjugates, can be applied to the sample. Proceed with the detection protocol above.

In Situ Hybridization to mRNA in Paraffin-Embedded Tissues

5.1 Incubate paraffin-embedded tissue sections on a microscope slide or coverslip for at least 1 hour at 65°C (up to overnight) to firmly bind the sections to the slide.

5.2 Deparaffinize the sections by incubating in xylene two times, for 5 minutes each time, at room temperature.

5.3 Rehydrate sections by incubating the slide sequentially in:
- 100% ethanol twice for 3 minutes each time,
- 95% ethanol twice for 3 minutes each time,
- 80% ethanol once for 3 minutes,
- 70% ethanol once for 3 minutes,
- 60% ethanol once for 3 minutes,
- water, until sections are transparent.

5.4 Incubate the section in 0.2 M HCl for 20 minutes at room temperature. Remove the solution.

5.5 Apply 0.3% Triton® X-100 in PBS to the sample — using 2.5–3 mL/60 mm petri dish, 5 mL/100 mm petri dish or 40 mL/Coplin staining jar — and incubate for 15 minutes at room temperature.

5.6 Treat the sample with 5–30 µg/mL proteinase K in 0.1 M Tris-HCl, 50 mM EDTA, pH 8.0, for 30 minutes at 37°C. *The amount of proteinase K necessary for deproteination may vary according to the particular tissue used, and therefore must be determined empirically. This step may not be necessary when using oligonucleotide probes.*

5.7 To prevent excessive digestion, rinse the section in 0.2% glycine, then post-fix the sample for 5 minutes in freshly prepared 4% paraformaldehyde in PBS.

5.8 Rinse the sample in PBS, then acetylate by incubating for 10 minutes in freshly made 0.25% acetic anhydride in 0.1 M triethanolamine (prepared *immediately* prior to the acetylation reaction).

5.9 Prehybridize the sample for 2 hours at 37°C. *Prehybridization and hybridization solutions must be optimized for each probe/target pair.*

5.10 Hybridize the sample overnight according to standard procedures.

5.11 Wash the sample to remove unbound probe, according to standard procedures. *Exact wash conditions must be optimized for each probe/target pair.*

5.12 At this point, other secondary detection reagents, such as biotinylated antibody conjugates, can be applied to the sample. Perform ELF 97 detection as described above in *ELF Detection Protocol*.

References

1. U.S. Patent No. 5,316,906; **2.** Mol Biol Cell 4, 226a, abstract #1313 (1993); **3.** FASEB J 8, A1444, abstract #1081 (1994); **4.** *Gene and Chromosome Analysis, Part A*, K.W. Adolf, Ed., Academic Press, San Diego (1993); **5.** M.L. Pardue in *Nucleic Acid Hybridization: A Practical Approach*, B.D. Hames and S.J. Higgins, Eds., IRL Press, Oxford, England (1985); **6.** Methods Enzymol 169, 741 (1989); **7.** A. Rashtchian in *Nonisotopic DNA Probe Techniques*, Larry Kricka, Ed., Academic Press, San Diego (1992).

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

Cat #	Product Name	Unit Size
E-6604	ELF® 97 mRNA *In Situ* Hybridization Kit #1 *50 assays*	1 kit
E-6605	ELF® 97 mRNA *In Situ* Hybridization Kit #2 *with streptavidin, alkaline phosphatase conjugate* *50 assays*	1 kit
E-6606	ELF® spin filters *20 filters*	1 box

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