



ELF[®] Phosphatase Detection Kit

For Embryonic Stem Cells

Optimized by the ATCC Stem Cell Center

Catalog no. SCRR-3010

Instruction Manual

Store at 2 to 8 °C. Protect from light. Do not freeze.

This product is for laboratory use only. Not for human diagnostic use.

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INTRODUCTION

Pluripotent stem cells, including embryonic stem (ES) cells, embryonal carcinoma (EC) cells and embryonic germ (EG) cells exhibit unique properties that have been exploited for stem cell derivation, purification, and analysis (1-3). The transcription factor protein Oct-3/4 and enzyme markers such as phosphatases have been investigated for their potential to identify and distinguish in vitro differentiation in ES cell populations.

Earlier methods to detect endogenous phosphatase activity in ES cell cultures resulted in adequate fluorescence but were time consuming. In the ELF detection assay, the weak blue fluorescence of the substrate is converted to intensely fluorescent yellow-green precipitate at the site of enzymatic activity. The ATCC Stem Cell Center optimized this method for use on stem cells and regularly uses it on fixed cells, allowing efficient and convenient phosphatase detection in ES cells (4).

The ELF phosphatase precipitate exhibits a fluorescence emission that is separated from its excitation wavelength by greater than 100 nm (Figure 1). The excitation/emission of the reaction product is 345/530 nm. Staining appears yellow-green against a blue background when visualized with a Hoechst/DAPI longpass filter set (usually supplied with fluorescence microscopes).

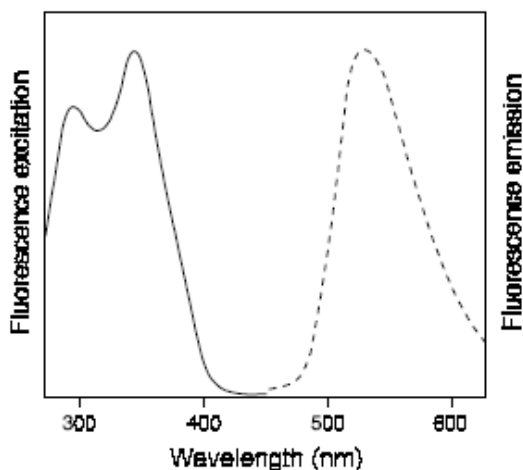


Figure 1 ■ The normalized excitation (—) and emission (---) spectra of the ELF alcohol precipitate generated by the enzymatic cleavage of the soluble ELF phosphatase substrate.

KIT COMPONENTS

Store all components at 2 to 8 °C. Protect from light. Do not freeze.

- ELF phosphatase substrate (Component A), 500 µl of 20X concentrate, containing 2 mM sodium azide
- Detection buffer (Component B), 10 ml
- Mounting medium (Component C), 15 ml

MATERIALS REQUIRED BUT NOT SUPPLIED

- Phosphate buffered saline (PBS),
- Wash buffer (PBS with 25 mM EDTA and 5 mM levamisole, pH 8.0)
- Micropipettors
- 0.2-µm spin or syringe filters (optional)
- Fluorescent microscope with DAPI/Hoechst longpass filter set

The phosphatase 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

Note: Read through the entire protocol and assemble all necessary buffers and reagents before starting. Because the reaction with the phosphatase enzymes is very fast and delays may result in inferior results, the microscope should be fitted with the appropriate filter sets for viewing the ELF precipitate before starting the protocol.

1. Fix the cells or tissue cells or tissue according to standard techniques.

The quality of the signal is dependent on the fixation protocol, which must be determined empirically for each sample. In general, 4% formaldehyde is a suitable fixative, but the fixation time may vary. Cells grown on coverslips may be fixed in 4% formaldehyde in phosphate buffered saline (PBS) for 10 to 15 minutes at room temperature and then rinsed in PBS. Acetone and ethanol are also suitable fixatives. **Avoid methanol** because it often inactivates alkaline phosphatase activity.

The method may be applied on live cells, but the effects on cell morphology and cell viability have not been characterized.

2. 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 (5).

3. Rinse in PBS for at least 10 minutes.

4. Dilute the Phosphatase Substrate (Component A) 20-fold in Detection Buffer (Component B).

Prepare only the amount of diluted substrate solution required for the day's experiments; 20 to 200 μ l will be used for each sample. The 20-fold dilution 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 at the lowest concentration possible.

Too high a concentration will result in overlabeling (a granular appearance) and possibly high background (green fluorescent crystals all over the sample). If too low 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.

5. Filter the diluted substrate solution through a 0.2- μ m filter just before applying to tissue sections.

The filtering step is required to remove any aggregates of the substrate that may have formed during storage, which may form spurious crystals in the sample. Using spin filters in a microcentrifuge tube will allow a very small volume to be filtered without significant loss of sample. The solution is added to the spin filter and then centrifuged in a microcentrifuge for 10 to 30 seconds. For filtering large volumes we recommend using a syringe filter.

6. Apply the substrate solution while at the microscope.

Before applying the substrate solution, make sure that you have the appropriate filter sets installed in your microscope. The signal can be seen through a DAPI or Hoechst longpass filter set or using a custom filter set. If you can easily reconfigure your filter sets, a custom filter set can be obtained by using 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 signal.

The reaction occurs within 30 to 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, wick or shake off any excess PBS from the sample. Apply 20 to 50 μ l of the filtered substrate solution directly to the sample or to the coverslip (which will be inverted and gently placed 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.

The reaction is usually complete within 30 to 90 seconds and rarely requires longer than 5 minutes. Carefully monitor the labeling reaction to generate the best detection. The yellow-green fluorescent precipitate is very photostable and will withstand long periods of visualization. Cleavage of the phosphatase 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 be visible.

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 reactions 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.

7. Stop the reaction by submerging the sample in wash buffer (PBS with 25 mM EDTA and 5 mM levamisole, pH 8.0).

The pH of the wash buffer must not be above 8.0. The signal may dissolve at higher pH.

Levamisole is used as a specific inhibitor of alkaline phosphatase activity in the mammalian liver/bone/kidney family. In contrast, intestinal alkaline phosphatases are not generally inhibited by levamisole but can be inhibited with phenylalanine (6).

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

Wash the sample completely. Excess phosphatase substrate left on the sample may form background crystals.

9. Remove as much wash buffer as possible without drying the sample and mount in the Mounting Medium (Component C).

If pools of liquid are left on the sample, crystals may develop over time. Leave mounted slides on a flat surface overnight to dry. The mounting medium will set in a few hours, making it possible to store the slides upright in a slide box at room temperature. Seal the coverslip to the slide with melted wax to store slides indefinitely.

The mounting medium included in the kit has been specially formulated to preserve the resolution of the signal. Other mounting media may cause the signal to degrade with time.

10. Visualize the stained sample.

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 precipitate will not be visible.

INTERPRETATION OF RESULTS

The alkaline phosphatase substrate will fluoresce bright yellow-green, indicating which cells are pluripotent (Figures 2 and 3). The absence of fluorescence indicates differentiation. Uneven fluorescence within a colony indicates that some cells in the population have begun to differentiate.

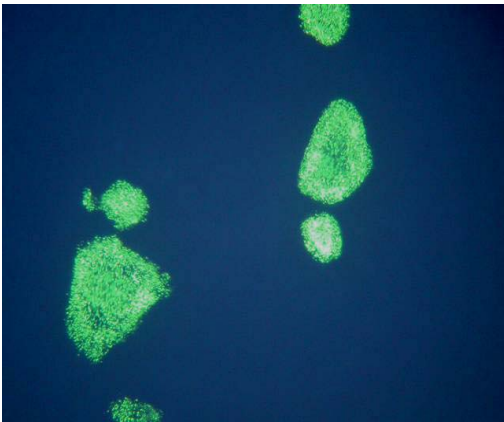
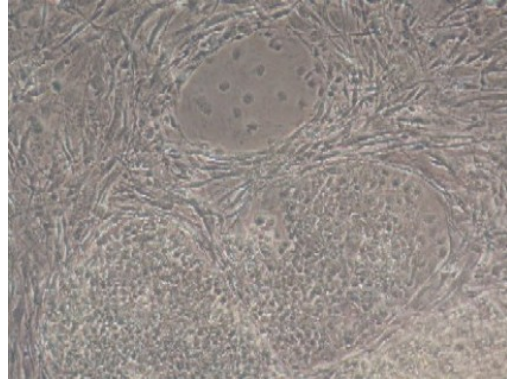
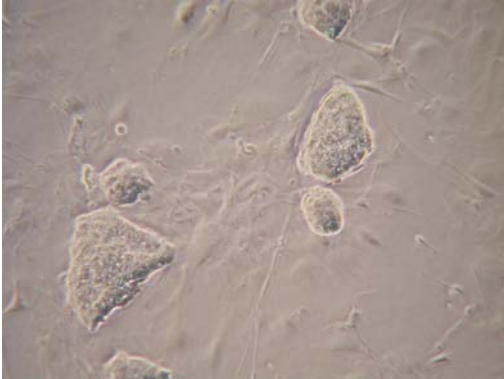


Figure 2 ■ Phosphatase activity in undifferentiated mouse R1 stem cell colonies.

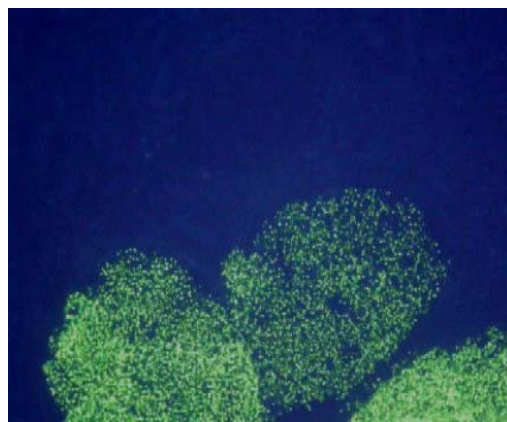


Figure 3 ■ Phosphatase activity in human embryonic stem cell colonies.

STAINING WITH MULTIPLE DYES

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

TROUBLESHOOTING

Problem: The fluorescence is too dim (underlabeled).

Possible solutions:

- Prepare fresh fixation solution on the day of the assay.
- Permeabilize the cells with Tween[®] 80 (see step 2).
- Increase the concentration of phosphatase substrate (step 4).
- Allow the labeling reaction to proceed for a longer time (step 6).
- Make sure the pH of the wash buffer is not above 8.0 (step 7).
- Perform a conventional phosphatase detection test in parallel to verify enzyme activity.

Problem: The fluorescence is too strong (overlabeled).

Possible solutions:

- Prepare fresh fixation solution on the day of the assay.
- Decrease the concentration of phosphatase substrate (step 4).
- Add the substrate at the microscope and monitor the reaction closely to ensure that it stops before overlabeling occurs (step 6).

Problem: Background fluorescence interferes with viewing.

Possible solutions:

- Decrease the concentration of phosphatase substrate (step 4).
- Add the substrate at the microscope and monitor the reaction closely to ensure that it stops before overlabeling occurs (step 6).
- Wash the slide thoroughly after the stop solution has been added (step 8).

Problem: Fluorescent crystals appear in sample.

Possible solutions:

- Filter the phosphatase substrate solution before use (step 5).
- Add the substrate at the microscope and monitor the reaction closely to ensure that it stops before overlabeling occurs (step 6).

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FREQUENTLY ASKED QUESTIONS

1. The kit lists Levamisole as a required reagent. Where can I get it?

The ATCC Stem Cell Center recommends Levamisole Hydrochloride from VWR, Catalog# TCT1215-010G, also listed as Tetramisole Hydrochloride and supplied by TCI America.

2. Do you recommend a specific type of filter for use with the ELF substrate?

The ATCC Stem Cell Center commonly uses 0.2 µm cellulose acetate syringe filters.

3. What should I do if I do not see the bright yellow-green result 5 minutes after adding the substrate?

Do not stop the reaction. Give the reaction another 5 or 10 minutes. The time at which the reaction becomes visible is highly affected by the colony morphology. Using the same concentration of substrate, three dimensional mouse embryonic stem cell colonies produce a visible result faster than human embryonic stem cells that grow in monolayers. The 1:20 dilution is a suggested value, but may be altered to produce a more rapid result. However, be careful not to make the solution too concentrated, which would increase the background staining. It is best to determine the proper dilution and reaction time for your particular cell line.

4. I just fixed the cells in 4% paraformaldehyde and have to leave the lab. Can I put off the rest of the protocol until a later time?

We recommend performing the assay on the same day you fix the cells. However, you can perform the assay the next day if you wash the fixed cells and store them at 4°C in 1X PBS. Do not exceed 24 hours.

5. When or how often should this assay be used on embryonic stem cells?

Embryonic stem cell lines are more sensitive to culture conditions than other cell lines and exhibit a tendency to differentiate. The ATCC Stem Cell Scientists recommend running this assay before starting an experiment wherein undifferentiated stem cells are required. It is also recommended that you run this assay anytime you have expanded the culture significantly or notice an unexpected change in morphology.

6. Are there other markers for undifferentiated embryonic stem cells?

Yes, OCT 3/4 is another marker commonly used by the ATCC Stem Cell Center to detect undifferentiated human and mouse embryonic stem cells. The ATCC Stem Cell Scientists also use SSEA-1 as a marker for mouse embryonic stem cells and SSEA-4 as a marker for human embryonic stem cells. The nice thing about the alkaline phosphatase method in this kit, is that it is convenient, cost effective, and provides a result similar to a “yes” or “no” response.

RELATED PRODUCTS

The ATCC Stem Cell Center distributes well-characterized stem cells from a variety of sources and offers support products such as feeder layer cells, media, sera, and reagents that are optimized for use with stem cells. For more information, visit stemcells.atcc.org.

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