3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

PKH2 Green Fluorescent Cell Linker Kit for General Cell Membrane Labeling

Catalog Number **PKH2GL**

TECHNICAL BULLETIN

Product Description

The PKH2-GL cell linker kit uses patented membrane labeling technology to stably incorporate a fluorescent dye with long aliphatic tails (PKH2) into lipid regions of the cell membrane.[1] The labeling vehicle provided with the kit (Diluent A) is an iso-osomotic aqueous solution which contains no salts, detergents or organic solvents and is designed to maintain cell viability while maximizing dye solubility and staining efficiency. The pattern of staining is dependent upon the cell type being labeled and the membrane of the cells.[2,3] PKH2, a green fluorescent cell linker (Figure 1), has been characterized in a wide variety of systems and found to be useful for *in vitro* and *ex vivo* cell labeling[2,3], *in vitro* cell proliferation studies[4,5], and *in vitro* and *in vivo* cell tracking applications[2-8].

Components

- PKH2 dye stock (1 vial containing >0.5 ml, 1 x 10^{-3} M in ethanol)
- Diluent A (6 vials, each containing >10 ml)

Storage/Stability

- Dye stocks may be stored at room temperature or refrigerated, protected from the light, and must be examined for crystals prior to use. If crystals are noted in the dye stock, it should be warmed slightly in a 37 °C water bath, and/or sonicated to redissolve crystals. Because the dye is in ethanol, the dye stock **must be kept tightly capped** when not in immediate use to prevent evaporation.
- The diluent may be stored at room temperature or refrigerated; however, note that it does not contain any preservatives or antibiotics and should be kept sterile.
- Working solutions of dye in Diluent A should be made **immediately** prior to use.
- Do not store the dye in diluent.

Procedures

- Materials needed for General Membrane Labeling but not included:
- A uniform single cell suspension of cells in tissue culture medium.
- Tissue culture medium with serum.
- Tissue culture medium without serum, or Dulbecco's PBS (Ca^{2+} and Mg^{2+} free).
- Serum, albumin or other system-compatible protein source.
- Polypropylene conical centrifuge tubes.
- Temperature-controlled centrifuge (0 - 1000 x *g*).
- Instrument(s) for analysis of fluorescence (fluorometer, fluorescence microscope, flow cytometer, or fluorescence image analysis instrumentation).
- Laminar flow hood.
- Hemocytometer or cell counter.
- Slides and coverslips.

General Cell Membrane Labeling

The appearance of labeled cells may vary from bright and uniform labeling to a punctuate or patchy appearance. Because the labeling is not a saturation reaction but a function of both dye and cell concentration, it is essential that the amount of dye available for incorporation be limited. Over-labeling of the cells will result in loss of membrane integrity and reduced cell recovery.

The sample cell and dye concentrations given in this procedure are the starting concentrations broadly applicable to a variety of cell types. To maximize fluorescence per cell, users must determine the optimum dye/cell concentration for their cell type(s) and experimental purposes (See Figure 2, page 4). Also, the user should evaluate cell viability (e.g., propidium iodide exclusion), fluorescence intensity, coefficient of variation of fluorescence peaks, and uniformity of staining.[9]

To stain at final concentrations of 2 x 10^{-6} M PKH2 dye and 1 x 10^{7} cells/ml in a 2 ml total volume, perform the followingusing aseptic techniques:

1. Adherent or bound cells must first be removed using proteolytic enzymes (i.e., trypsin/EDTA) and put into a single cell suspension.

2. Perform all steps at 25 °C. Place a total of approximately 2 x 10^7 single cells in a conical bottom polypropylene tube and wash once using medium without serum.
3. Centrifuge the cells (400 x *g*) for 5 minutes into a loose pellet. .
4. After centrifuging cells, carefully aspirate the supernatant leaving no more than 25 µl of supernatant on the pellet.
5. Add 1 ml of Diluent A. . Resuspend cells, pipetting to insure complete dispersion. Do not vortex.
6. **Immediately** prior to staining, prepare 4 x 10^{-6} molar PKH2 dye (this will be a 2x stock) in polypropylene tubes using Diluent A (supplied with the kit). To minimize ethanol effects, the amount of dye added should be less than 1% of the individual sample volume. If a greater dilution of the dye stock is necessary, make an intermediate stock by diluting with 100% ethanol. The preparation should remain at room temperature (25 °C).
7. Rapidly add the 1 ml of 2x cells to 1 ml of 2x dye. **Immediately** mix the sample by pipetting. Rapid and homogeneous mixing is critical for uniform labeling because staining is nearly instantaneous.
8. Incubate at 25 °C for 2-5 minutes. Periodically, invert the tube gently to assure mixing during this staining period at 25 °C.
9. Stop the staining reaction. Add an equal volume of serum or compatible protein solution (i.e., 1% BSA). Incubate 1 min.
10. Dilute the serum-stopped sample with an equal volume of complete medium. Do **not** use Diluent A.
11. Centrifuge the cells at 400 x *g* for 10 minutes at 25 °C to remove cells from staining solution.
12. Remove the supernatant and transfer the cell pellet to a new tube for further washing (a minimum of 3 washes is recommended).
13. Add 10 ml of complete medium to wash the cells, centrifuge and resuspend the cells to the desired concentration.
14. Examine the cells using fluorescence microscopy, flow cytometry or fluorescence-based image analysis. The stained sample should be checked for cell recovery, cell viability, and fluorescence intensity. Staining should be uniform and is typically 100-1,000 times brighter than background autofluorescence.

Critical Aspects of General Cell Membrane Labeling

1. Dye stocks may be stored at room temperature or refrigerated, and must be examined for crystals prior to use. If crystals are noted in the dye stock, it should be warmed slightly in a 37 °C water bath, and/or sonicated to redissolve crystals. Store protected from light. Because the dye is in ethanol, **the dye stock must be kept tightly capped when not in immediate use to prevent evaporation.**
2. The diluent may be stored at room temperature or refrigerated; however, note that it does not contain any preservatives or antibiotics and should be kept sterile.
3. Starting solutions of dye should be made **immediately** prior to use. Do not store the dye in Diluent A.
4. No azide or metabolic poisons should be present at the time of PKH2 staining.
5. It is imperative that single cell suspensions be used, to obtain uniform staining.
6. Prior to staining remove all serum proteins and lipids that may reduce the effective dye concentration available for labeling.
7. The presence of salt causes micelle formation of the dye and interferes with the staining reaction. It is important that the cells be resuspended in Diluent A prior to the addition of dye. Therefore, dye stock should not be added directly into the cell suspension nor should dye solution be added directly to the cell pellet.
8. Rapid and homogeneous mixing is critical for uniform labeling. The following measures have been found to aid in achieving optimum results:
   a) Mix equal volumes of cell suspension and working dye solution;
   b) Avoid staining in very small (<100 µL) or very large (>5 mL) volumes or use of serological pipettes for the addition of cells to dye;
   c) Dispense volumes as precisely as possible so that both cell and dye concentrations are accurately reproduced from sample to sample.
9. The dye and diluent should be applied to the cells for as short a time as possible. Additional exposure may have some toxicity toward specific cell types. To evaluate the diluent effects, expose cells to the diluent alone using the same procedure used for staining. Then, evaluate these cells for functional impairment.
10. Staining should be stopped by adding an equal volume of serum or other suitable protein source. Serum is preferred. Do not centrifuge the cells in Diluent A before stopping the staining reaction. Washing efficiency can be increased if serum proteins or albumin are added to the stop and washing solutions.
11. After removing the cells from Diluent A and dye, they should be transferred to a new tube and washed 3 times using medium. Do not use Diluent A.

12. This labeling procedure can be used for *in vitro* or *ex vivo* labeling of stem cells, lymphocytes, monocytes, endothelial cells or any other cell type where partitioning of dye into lipid regions of the cell membrane is desired. Alternate methods may be more suitable for *in vivo* labeling.
13. General cell labeling should be performed prior to monoclonal antibody staining. The cell tracking probes will remain stable during the monoclonal staining at 4 °C; however, capping of the monoclonal antibodies is highly probable if the general cell labeling (25 °C) is carried out subsequent to antibody labeling.
14. Stained cells may be fixed with 2% paraformalde-hyde and are stable for up to 3 weeks.
15. Platelet labeling requires a modification of this protocol.[2,8]

Histology

Preparation and preservation of slides containing PKH-labeled cells requires frozen tissue sectioning and special mounting techniques. Drs. Per Basse and Ronald H. Goldfarb (Pittsburgh Cancer Institute, Pittsburgh, PA) developed the following methods specifically for use with PKH fluorescent cell linker dyes.

Preparation of slides

1. Excise tissues to be sectioned and freeze immediately on dry ice.
2. Store tissues at !70 °C prior to sectioning.
3. Mount frozen tissues using O.C.T. compound (Tissue-Tek; Miles, Inc.)
4. Prepare 4 to 5 micron tissue sections.
5. Air dry slides for at least 1 hr. at room temperature.
6. Mount coverslip using 1-2 drops of cyanoacrylate ester glue. (Successful results have been obtained using the following brands of cyanoacrylate ester glue: Elmer=s Wonder Bond, Archer Instant Bonding Adhesive, Bondo Super Glue, Duro Super Glue, Scotch Instant Glue and Instant Krazy Glue).
7. Examine or photograph sections using standard filter setup for FITC (PKH2 & PKH67) or TRITC (PKH26).

Counterstain Sections

1. Remove coverslips by soaking slides in acetone for 24-48 hour.
2. Rinse slides in distilled water to remove acetone.
3. Counterstain sections using stain of choice. Satisfactory results have been obtained using Mayer s or Harris hematoxylin.
4. Mount slides using AS/AP permanent aqueous mounting medium (Bio/Can America, Inc., Portland, ME).

Note: Because organic solvents may extract PKH dyes and counterstains may absorb fluorescence, simultaneous visualization of PKH fluorescent cell linker dyes and histological staining has not been demonstrated. Use serial sections or use a single section and perform fluorescent microscopy before demounting and counterstaining.

References

1. Horan, P. and Slezak, S., Nature, **340**, 167-168 (1989).
2. Horan, P.K., et al., Methods Cell Biol., **33**, 469-490 (1990).
3. Poon, R.Y., et al., **In**: In Living Color: Flow Cytometry and Cell Sorting Protocols. (Diamond, R.A., DeMaggio, S. eds.). New York: Springer-Verlag, p. 302-352 (2000).
4. Boyd, F.T., Cell Growth Differ., **4**, 777-784 (1993).
5. Waters, W.R., et al., Immunology, **202**, 1-13 (2000).
6. Holloway, W., et al., Cytometry, **37**, 171-177 (1999).
7. Flieger, D., et al., J. Immunol. Meth., **180**, 1-13 (1995).
8. Michelson, A.D., et al., Proc. Natl. Acad. Sci. USA, **93**, 11877-11882 (1996).
9. Wallace, P.K., et al., Cancer Res., **53**, 2358-2367 (1993).

Distributed for Phanos Technologies.

Figure 1. PKH2 Excitation and Emission Spectra

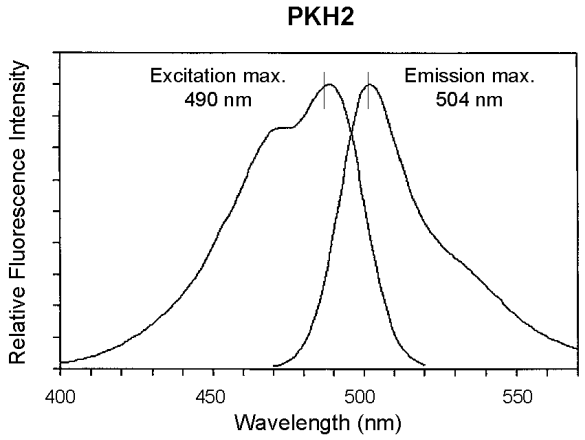
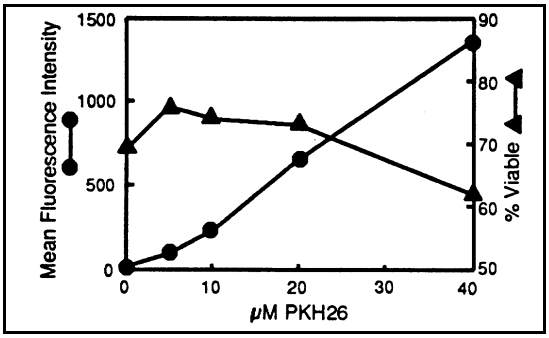


Figure 2. Staining Optimization for PKH Dyes
PKH2 staining concentration may be optimized using a method similar to that described for PKH26.

MC-38 TIL were stained with the indicated concentrations of PKH26 dye at a final cell concentration of 1 x 10^7 cells/ml. Viability ( ) was determined by trypan blue exclusion and mean fluorescence intensity (●) was determined from the flow cytometric histograms.
Reprinted with permission from the American Association for Cancer Research. Originally appeared in Cancer Research, ***53****, 2360 (1993).*



AH,PHC 08/10-1