Stock No. PKH26-GL
PKH26 Red Fluorescent Cell Linker Kit
For General Cell Membrane Labeling

The PKH26-GL fluorescent cell linker compound uses patented fluorescent cell linker technology to incorporate aliphatic reporter molecules into the cell membrane by selective partitioning.[1] The pattern of staining is dependent upon the cell type being labeled and the membrane of the cells.[2] PKH26, the red fluorescent cell linker, has been characterized in a limited number of model systems and has been found to be useful for *in vitro* cell labeling and *in vitro* and *in vivo* cell tracking applications.[3,4,5,6] The half-life of elution of PKH26 from rabbit red blood cells is greater than 100 days *in vivo*. This enhanced stability is favorable for long term *in vivo* studies.

Materials Required for General Cell Labeling

1. A uniform single cell suspension of cells in tissue culture medium.
2. Tissue culture medium with serum.
3. Tissue culture medium without serum, or Dulbecco's PBS (Ca^{2+} and Mg^{2+} free).
4. PKH26-GL Fluorescent Cell Linker Kit composed of PKH26 dye stock (1 vial containing >0.5 ml, 1 x 10^{-3} M) and Diluent C (6 vials, each contains >10 ml).
5. Serum, albumin or other system-compatible protein source.
6. Polypropylene conical centrifuge tubes.
7. Temperature-controlled centrifuge (0 - 1,000 x g).
8. Instrument(s) for analysis of fluorescence (fluorometer, fluorescence microscope, flow cytometer, or fluorescence image analysis instrumentation).
9. Laminar flow hood.
10. Hemocytometer or cell counter.
11. Slides and coverslips.

Methods for General Cell Labeling

The appearance of labeled cells may vary from bright and uniform labeling to a punctate 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 cell recovery.
The 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, page 3). Also, the user should evaluate cell viability (e.g., propidium iodide exclusion), fluorescence intensity, coefficient of variation of fluorescence peaks, and uniformity of staining.[7]

Concentration of the dye stock is 1 x 10^{-3} M in ethanol. To obtain a final concentration of 2 x 10^{-6} M PKH26 dye and 1 x 10^{7} cells/ml of Diluent C in a 2 ml volume, perform the following using 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. Wash a suspension of single cells once using medium without serum.
3. Centrifuge the cells (400 x g) for 5 minutes into a loose pellet. The total number of cells should be approximately 2 x 10^{7} per sample.
4. After centrifuging cells, carefully aspirate the supernatant leaving no more than 25 µl of supernatant on the pellet.
5. Tap the button of cells in the centrifuge tube to resuspend them in the residual medium in the tube, then add 1 ml of Diluent C to resuspend cells. Do not vortex.
6. **Immediately** prior to staining, prepare 4 x 10^{-6} molar PKH26 dye (this will be a 2x stock) in polypropylene tubes using Diluent C (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 gentle pipetting or by inverting the capped tube. Rapid and homogeneous mixing is critical for uniform labeling.
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 C.
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 distinguishable from background autofluorescence.

Critical Aspects of General Cell 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 C.
4. No azide or metabolic poisons should be present at the time of PKH26 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. It is important that the cells be resuspended in Diluent C prior to the addition of dye. Dye stock should not be added directly into the cell suspension nor should dye solution be added directly to the cell pellet. The presence of salt causes micelle formation of the dye and interferes with the staining reaction.
8. Rapid and homogeneous mixing is critical for uniform labeling. Use of equal volumes for cell suspensions and working dye soluutions and avoidance of very large or very small volumes is desirable to obtain optimum results.
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 C 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 C and dye, they should be transferred to a new tube and washed 3 times using medium. Do not use Diluent C.
12. The general cell labeling procedure can be used for monocytes, macrophages, lymphocytes, or any other cell type where general membrane labeling is desired. It is an *in vitro* procedure and has not been fully characterized 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% paraformaldehyde and are stable for up to 3 weeks.
15. Platelet labeling requires a modification of this protocol [Horan, P., et al., Meth. Cell Biol., **33**, 469 (1990)].

Histology Methodology[8]

Preparation and preservation of slides containing PKH-labeled cells requires frozen tissue sectioning and special mounting techniques. The following methods have been developed 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.

Storage

All reagents may be stored at room temperature or refrigerated.

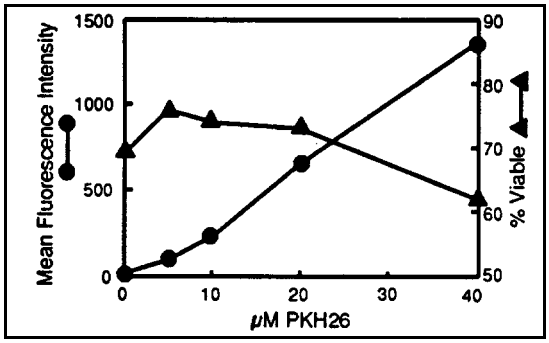
References

1. Horan, P., and Slezak, S., Nature, **340**, (6229), 167 (1989).
2. Horan, P., et al., Meth. Cell Biol., **33**, 469 (1990).
3. Slezak, S., and Horan, P., Blood, **74**, (6), 2171 (1990).
4. Samlowski, W., et al., J. Immunol. Methods, **144**, 101 (1991).
5. Hugo, P., et al., Nature, **360**, 679 (1992).
6. Messina, L., et al., Proc. Nat. Acad. Sci. (USA), **89**, (24), 12018 (1992).
7. Wallace, P., et al., Cancer Res., **53**, 2358 (1993).
8. This method was developed by Drs. Per Basse and Ronald H. Goldfarb, Pittsburgh Cancer Institute, Pittsburgh, PA.

US Patent Numbers: 4,783,401; 4,762,701; 4,859,584.
Distributed for Phanos Technologies.

PKH26 Excitation and Emission Spectra
Excitation max. = 551 nm, Emission max. = 567 nm

Staining Optimization of PKH Dye



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).*

TROUBLE SHOOTING GUIDE FOR PKH2, PKH26 AND PKH67 FLUORESCENT CELL LINKER DYES

	Problem	**Solution**
Cell Clumping	Dye concentration too high.	Lower labeling concentrations.
	Serum not added to stop reaction.	Serum, other protein or complete medium must be used to stop labeling reaction.
	Platelets present in whole blood sample.	Centrifuge sample at low speed to remove platelets before staining.
	Adherent cells not disaggregated.	Mechanical (aspirate through needle) or enzymatic (trypsin) digestion prior to staining.
	Poor initial cell viability of sample.	Incubate with 0.002% DNase for 30 minutes at 37°C. before staining.
	Cells exposed to dye too long.	Expose cells to dye for 2-5 minutes.
Poor Staining Intensity	Filters incorrect for observing dye.	Check filter set-up. Check cell pellet after labeling; if pellet is pink or yellow, cells are stained.
	Working dye stock prepared too long before adding to cells - dye aggregating.	Prepare dye immediately prior to labeling.
	Salt content of labeling solution too high - dye aggregating.	Centrifuge cells and remove as much supernatant as possible to minimize physiological salts after suspension in Diluent C.
	Serum present during labeling interfering with dye incorporation.	Wash cells in serum-free buffer 1-2 times prior to resuspension in Diluent C for labeling.
	Staining intensity varies with cell type based on cell size/surface area, lipid to protein ratios of membranes and lipid component of membranes.	Adjust dye concentration for each cell type and experimental application Analyze sub-populations independently.
	Dye concentration too low.	Increase dye concentration.
	Cell concentration too high.	Reduce cell concentration to a range of 10^6 to 10^9 cells/ml.
Patchy or Punctate Staining	Salt content of labeling solution too high - dye aggregating.	Centrifuge cells and remove as much supernatant as possible to minimize physiological salts after suspension in Diluent C.
	Some localization of dye on certain cell membranes causes a patchy appearance.	May be cell type dependent and not fixable.

TROUBLE SHOOTING GUIDE FOR PKH2, PKH26 AND PKH67 FLUORESCENT CELL LINKER DYES continued		
	Problem	**Solution**
Hetero-geneous Staining	Different cell types in sample - staining intensity varies from cell type to cell type.	Isolate desired cell types prior to labeling - perform analysis on one cell type only.
	Heterogeneous exposure to dye during staining.	Ensure rapid and homogeneous mixing at the time of dye addition . Optimize order of addition and method of mixing.
	Dye concentration too low.	Increase dye concentration.
	Cell concentration too high.	Lower cell number per ml, use a range of 10^6 to 10^9 cells/ml.
	Serum present during labeling interfering with dye incorporation.	Wash cells in serum-free buffer 1-2 times prior to resuspension in Diluent C for labeling.
	Dye sticking to test tube walls.	Use only polypropylene tubes, other plastics absorb dye.
	Cells clumped - not single cells in suspension.	Carefully suspend cells in media - use aspiration through a needle to break clumps.
Cells Dead	Dye concentration too high.	Lower dye concentration.
	Serum used to stop reaction was not heat inactivated.	Heat serum at 56°C for 60 minutes.
	Sensitivity to diluent.	Run diluent only control - monitor viability.
Viability/ Function	Poor viability or recovery. Functional alteration of cells.	Over incorporation of dye molecules (over-labeling) will affect cells - adjust (lower) dye concentration and/or (increase) cell concentration.
	Difficulty resuspending pelleted cells because of clumping in bottom of test tube.	Over incorporation of dye molecules (over-labeling) will affect cells - adjust (lower) dye concentration and/or increase cell concentration. Incubate cells with 0.002% DNase for 30 minutes at 37°C after stopping staining and before centrifuging.
	Cells appear shriveled when visualized in microscope.	Non-mammalian cells may require special diluent formulations - Call Sigma Technical Service.
Dye Leakage/ Transfer	Dye transfer between cells in co-culture.	Wash cells 3-5 times after labeling - transfer sample to new tubes between washes. PKH2 tends to transfer more readily between cells in co-culture - try PKH67 or PKH26.
	Leakage of dye from target cells in cytotoxicity assays.	PKH2 tends to transfer more rapidly from certain cell types (e.g. K562, NS-1 cell lines) - try PKH67 or PKH26.
	Organic based solvents or detergents will extract dye.	Use only aqueous-based fixatives and solvents.
	Phagocytosis of target cells by effector cells.	Monitor for phagocytosis by microscopy.
	Transfer occurs initially, but stops over time.	Culture in medium for several hours prior to mixing with unlabeled cells [Embleton, M.,et al., Int. J. Cancer, 49, 566 (1991)].
Fixation of tissue sections	Frozen sections show no fluorescence.	Use only dry ice to freeze tissue for sectioning - other solutions (i.e. isopentane, DMSO) will extract dye.
	Low fluorescence intensity.	Be sure sections are dry before adding glue - allow glue to dry. Check autofluorescence of tissue and then select appropriate cell linker dye.
	Counterstaining absorbs fluorescence.	Use serial sections or use a single section and perform microscopy before demounting and counterstaining for morphology.

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