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Product Information

PKH67 Fluorescent Cell Linker Kits
For General Cell Membrane Labeling

Catalog Numbers **MINI67, MIDI67, and PKH67GL**
Store at Room Temperature

TECHNICAL BULLETIN

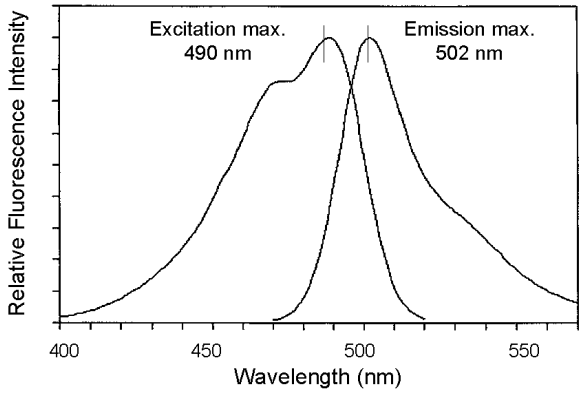
Product Description

The PKH67 Fluorescent Cell Linker Kits use proprietary membrane labeling technology to stably incorporate a green fluorescent dye with long aliphatic tails (PKH67) into lipid regions of the cell membrane.[1,2] The labeling vehicle provided in the kits (Diluent C) is an aqueous solution designed to maintain cell viability, while maximizing dye solubility and staining efficiency during the labeling step.[3] Diluent C is iso-osmotic for mammalian cells and contains no detergents or organic solvents, but also lacks physiologic salts and buffers.[1] The appearance of labeled cells may vary from bright and uniform to punctate or patchy, depending on the cell type being labeled and the extent to which membrane internalization occurs after labeling.[2-5] However, PKH67 fluorescence is independent of pH within physiologic ranges and fluorescence intensity per cell is typically unaffected by the pattern of dye localization.[1,4,5]

PKH67 fluoresces in the green (Figure 1). Due to its longer aliphatic carbon tails, PKH67 exhibits reduced cell-cell transfer [6] compared with its predecessor, PKH2.[2,3] PKH67 is well suited for cytotoxicity assays that use propidium iodide or 7-aminoactinomycin D as viability probes[3,7-11] or for use in combination with orange-red fluorescent probes such as phycoerythrin, red fluorescent proteins, *etc.* PKH67 is often used for proliferation monitoring based on dye dilution,[12-18] including estimation of antigen-specific precursor frequencies[14-16] and identification of quiescent/slowly dividing tumor cells with stem-like properties.[17,18] It has also proven useful for monitoring exosome[19] or liposome[20] uptake, cell-cell membrane transfer,[21-24] phagocytosis, [25,26] and antigen presentation[19,22,24,26] as well as for *in vivo* cell trafficking studies.[27-29]

Correlation of *in vitro* cell membrane retention with *in vivo* rate of intensity decrease in non-dividing cells predicts an *in vivo* fluorescence half-life for PKH67 of 10–12 days.[30] This is similar to the *in vivo* half-lives of PKH1 and PKH2, which have successfully been used to monitor *in vivo* lymphocyte and macrophage trafficking for periods of 1–2 months.[31,32] PKH67 is therefore recommended for short-to-medium term *in vivo* studies requiring a green cell linker dye[27-29], as well as for *in vitro* cytotoxicity, phagocytosis, proliferation, antigen presentation, or other co-culture assays.[7-26] Due to its extremely stable fluorescence,[3] PKH26 remains the cell linker dye of choice for longer term *in vivo* studies in which labeled cells are to be followed for periods longer than a few weeks.[4,33-37]

Figure 1. PKH67 Excitation and Emission Spectra



Components

	PKH67 Dye* (P7333)	Diluent C (CGLDIL)
MINI67	1 × 0.1 mL	1 × 10 mL
MIDI67	2 × 0.1 mL	6 × 10 mL
PKH67GL	1 × 0.5 mL	6 × 10 mL

* 1×10^{-3} M in ethanol

The MINI67 kit is recommended for small or preliminary studies, the MIDI67 kit for *in vitro* proliferation or cytotoxicity studies, and the PKH67GL kit for *in vivo* studies.

Equipment and Reagents Required but Not Provided for General Membrane Labeling

- Uniform suspension of well-dispersed single cells in tissue culture medium
- Tissue culture medium with serum (complete medium)
- Ca^{2+}, Mg^{2+}, and serum free medium or buffered salt solution (e.g., Dulbecco's PBS or Hank's BSS)
- Serum, albumin, or other system-compatible protein
- Polypropylene conical bottom centrifuge tubes (4–15 mL)
- Temperature-controlled centrifuge (up to 1,000 × *g*)
- Instrument(s) for fluorescence analysis (fluorescence plate reader, fluorescence or confocal microscope, flow cytometer)
- Laminar flow hood
- Hemocytometer or cell counter
- Slides and coverslips

Precautions and Disclaimer

These products are for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The PKH67 ethanolic dye solution (Catalog Number P7333) may be stored at room temperature or refrigerated. To prevent increases in dye concentration due to evaporation, **keep the ethanolic dye solution tightly capped** except when in immediate use. The dye solution must be protected from bright direct light and examined for crystals prior to use. If crystals are noted in the dye solution, warm slightly in a 37°C water bath, and sonicate or vortex until redissolved.

Diluent C may be stored at room temperature or refrigerated. If refrigerated, bring to room temperature before preparing cell and dye suspensions for labeling (Procedures, steps A5 and A6). Diluent C is provided as a sterile solution. Because it does not contain any preservatives or antibiotics, it should be kept sterile. **Do not store dye in Diluent C.** Working solutions of dye in Diluent C should be made **immediately** prior to use.

Procedures

A. General Cell Membrane Labeling.[1-4,10]

Labeling occurs by partitioning of the lipophilic dye into cell membranes. Labeling intensity is a function of both dye concentration and cell concentration and is not saturable. Therefore, it is essential that the amount of dye available for incorporation be limited. Over-labeling of cells will result in loss of membrane integrity and reduced cell recovery.

The following labeling procedure can be used for *in vitro* or *ex vivo* labeling of stem cells, lymphocytes, monocytes, endothelial cells, neurons or any other cell type where partitioning of dye into lipid regions of the cell membrane is desired.[1-29] Modified procedures may be required for *in vivo* labeling[39] and for labeling platelets or phagocytes.[2,3,40,41]

General cell membrane labeling should be performed prior to monoclonal antibody staining. The membrane dyes will remain stable during the monoclonal staining at 4 °C; however, capping of the monoclonal antibodies is highly probable if the general cell membrane labeling is carried out at ambient temperature subsequent to antibody labeling.

The cell and dye concentrations given in the following procedure represent starting concentrations that have been found broadly applicable to a variety of cell types.[7] **Users must determine the optimal dye and cell concentrations for their cell type(s) and experimental purposes** by evaluating post-staining cell viability (e.g., propidium iodide exclusion), fluorescence intensity, staining homogeneity, and lack of effect on cell function(s) of interest.[1,10,38]

Note 1 : No azide or metabolic poisons should be present at the time of staining with PKH67.

Note 2: Although adherent cells may be labeled while attached to a substrate, more homogeneous staining is obtained using single cell suspensions. Best results will be obtained if adherent or bound cells are dispersed into a single cell suspension using proteolytic enzymes, e.g., trypsin/EDTA, prior to staining.

The following procedure uses a 2 mL final staining volume containing final concentrations of 2×10^{-6} M of PKH67 and 1×10^{7} cells/mL.

Perform all further steps at ambient temperature (20–25 °C)

1. Place a suspension containing 2×10^{7} single cells in a conical bottom polypropylene tube and wash once using medium without serum.

Note: Serum proteins and lipids also bind the dye, reducing the effective concentration available for membrane labeling. Best results are obtained by washing once with serum-free medium or buffer (step 2) prior to resuspension in Diluent C for labeling (step 4).

2. Centrifuge the cells ($400 \times g$) for 5 minutes into a loose pellet.

Note: The PKH67 ethanolic dye solution should not be added directly to the cell pellet. This will result in heterogeneous staining and reduced cell viability.

3. After centrifuging cells, carefully aspirate the supernatant, being careful not to remove any cells but leaving no more than 25 µL of supernatant.

Note: For reproducible results, it is important to minimize the amount of residual medium or buffer present when cells are resuspended in Diluent C. See Note 28 in Ref. 10 for suggested methods.

4. Prepare a 2× Cell Suspension by adding 1 mL of Diluent C (Catalog Number CGLDIL) to the cell pellet and resuspend with gentle pipetting to insure complete dispersion. **Do not vortex and do not let cells stand in Diluent C for long periods of time**.

Note: The presence of physiologic salts causes the dye to form micelles and substantially reduces staining efficiency. Therefore, it is important that the cells be suspended in Diluent C at the time dye is added, not in medium or buffered salt solutions.

5. **Immediately prior to staining**, prepare a 2× Dye Solution (4×10^{-6} M) in Diluent C by adding 4 µL of the PKH67 ethanolic dye solution (Catalog Number P7333) to 1 mL of Diluent C in a polypropylene centrifuge tube and mix well to disperse.

Note 1: To minimize ethanol effects on cell viability, the volume of dye added in step 5 should result in no more than 1–2% ethanol at the end of step 6.

Note 2: If a final dye concentration $< 2 \times 10^{-6}$ M is desired, the most reproducible results will be obtained by diluting the PKH67 ethanolic dye solution provided in the kit with

100% ethanol to make an intermediate dye stock.

6. **Rapidly add** the 1 mL of 2× Cell Suspension (step 4) to 1 mL of 2× Dye Solution (step 5) and **immediately mix** the sample by pipetting. Final concentrations after mixing the indicated volumes will be 1×10^7 cells/mL and 2×10^{-6} M PKH67.

Note 1: Because staining is nearly instantaneous, **rapid and homogeneous dispersion of cells in dye solution is essential for bright, uniform and reproducible labeling**. The following measures have been found to aid in optimizing results:[1,10]

a. Do not add ethanolic PKH67 dye directly to the 2× Cell Suspension in Diluent C.
b. Mix equal volumes of 2× Cell Suspension (step 4) and 2× Dye Solution (step 5).
c. Adjust 2× cell and 2× dye concentrations to avoid staining in very small (<100 µL) or very large (>5 mL) volumes.
d. Use a Pipetman or equivalent for rapid addition of cells and admixing with dye. Serological pipettes are slower and give less uniform staining. Mixing by "racking" or vortexing is also slower and gives less uniform staining.
e. Dispense volumes as precisely as possible in order to accurately reproduce both cell and dye concentrations from sample to sample and study to study.

7. Incubate the cell/dye suspension from step 6 for 1–5 minutes with periodic mixing. Because staining is so rapid, longer times provide no advantage.

Note: Expose cells to dye solution and Diluent C for the minimum time needed to achieve the desired staining intensity. Since Diluent C lacks physiologic salts, longer exposure may cause reduced viability in some cell types. If such effects are suspected, include a diluent-only control and a mock-stained control using ethanol rather than dye.

8. Stop the staining by adding an equal volume (2 mL) of serum or other suitable protein solution (e.g., 1% BSA) and incubate for 1 minute to allow binding of excess dye.

Note 1: **Serum** (or an equivalent protein concentration) **is preferred as the stop solution**. Increase volume to 10 mL if complete medium is used instead of serum.

Note 2: Do not stop by adding Diluent C or centrifuge the cells in Diluent C before stopping the staining reaction.

Note 3: **Do not use serum-free medium or buffered salt solutions**, which cause formation of cell-associated dye aggregates. Dye aggregates act as slow-release reservoirs of unbound dye that are not efficiently removed by washing and can transfer to unlabeled cells present in an assay.

9. Centrifuge the cells at 400 × g for 10 minutes at 20-25 °C and carefully remove the supernatant, being sure not to remove cells. Resuspend cell pellet in 10 mL of complete medium, transfer to a fresh sterile conical poly-propylene tube, centrifuge at 400 × g for 5 minutes at 20-25 °C, and wash the cell pellet 2 more times with 10 mL of complete medium to ensure removal of unbound dye.

Note 1: Transfer to a fresh tube increases washing efficiency by minimizing carryover of residual dye bound to tube walls.

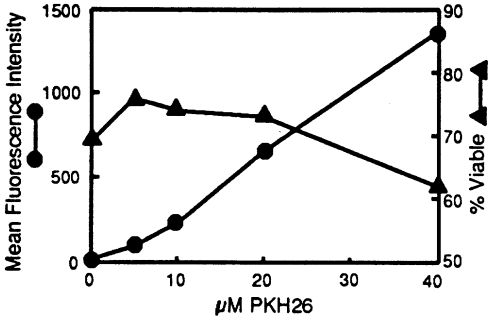
Note 2: Do not use Diluent C for washing steps.

10. After the final wash, resuspend the cell pellet in 10 mL of complete medium for assessment of cell recovery, cell viability and fluorescence intensity (**Figure 2**). Centrifuge and resuspend to desired final concentration of viable cells.

Note 1: Stained cells may be fixed with 1-2% neutral buffered formaldehyde and intensities are stable for at least 3 weeks if samples are protected from light.

Note 2: Staining is typically at least 100–1,000 times brighter than background autofluorescence. Intensity distributions should be symmetrical and as homogeneous as possible, although staining CV will depend on the cell type being stained.[4,10]

Figure 2. Staining Optimization for PKH67



PKH67 staining concentration may be optimized using a method similar to that described for PKH26.[35] MC-38 TIL cells were stained with the indicated concentrations of PKH26 dye at a final cell concentration of 1×10^7 cells/ml. Viability (▲) was determined by dye exclusion and mean fluorescence intensity (●) was determined by flow cytometry. Anti-tumor TIL specificity and potency *in vitro* and *in vivo* was confirmed to be unaltered by labeling with 20 µM PKH26.[35]
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B. Histology

PKH26 labeled adipocytes have been successfully identified in tissues subjected to standard paraffin embedding and sectioning,[34,] but such methods risk loss of intensity because they use clearing agents that may partially extract membrane lipids and lipophilic dyes.[1,3,4]

Histologic studies of tissues containing cells labeled with lipophilic membrane dyes have typically been carried out on serial frozen sections[38] or sections prepared after fixation in neutral buffered formalin prepared from 4% paraformaldehyde.[36,37,42] These methods avoid quenching of fluorescence by absorbing dyes found in histologic counterstains. For studies where imaging is to be done on a single section, fluorescence microscopy should precede counterstaining.[38]

The following methods, developed at the Pittsburgh Cancer Institute by Drs. Per Basse and Ronald H. Goldfarb for use with PKH26, are also expected to be useful for tissues containing cells labeled with PKH67.

Fluorescence imaging of frozen sections:

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.) or equivalent.
4. Prepare tissue sections.
5. Air dry slides for at least 1 hour 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 an appropriate filter setup (*e.g.*, FITC for PKH67).

Counterstaining of frozen sections:

1. Remove coverslips by soaking slides in acetone for 24–48 hours.
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) or equivalent.

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