PKH26 Cell Linker Kit

Cat #: B-CHK104

Size: 0.1 mL

Storage: Room temp

Components

Components	Amount
PKH26 Dye (1×10^{-3}M in EtOH)	0.1 mL
Diluent C	10 ml

Product Description

PKH26 Cell Linker Kit (for General Cell Membrane Labeling) is a test kit based on the fluorescent probe PKH26, used for general cell membrane labeling. It is suitable for in vitro cell labeling, in vitro cell proliferation, and long-term in vivo cell tracking research.

PKH26 is a patented membrane labeling probe with a long hydrophobic tail, which can stably insert into the lipid region of the cell membrane. PKH26 fluorescence is in the yellow-orange spectral range, with a maximum excitation wavelength of 551nm and a maximum emission wavelength of 567nm. It is compatible with Rhodamine or PE detection systems. It can also be excited with standard Fluorescein filters, but the fluorescence intensity may be slightly reduced. PKH26 has the longest in vivo half-life, exceeding 100 days, making it very suitable for in vivo cell tracking, cell proliferation research, and other long-term experiments, what's more, PKH26 dyes have been successfully used to label exosomes and extracellular vesicles in both in vitro and in vivo tracking experiments.

Materials Required but Not Supplied

- Uniform suspension of well-dispersed single cells in tissue culture medium
- Tissue culture medium with serum (complete medium)
- Ca2+, Mg2+, 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

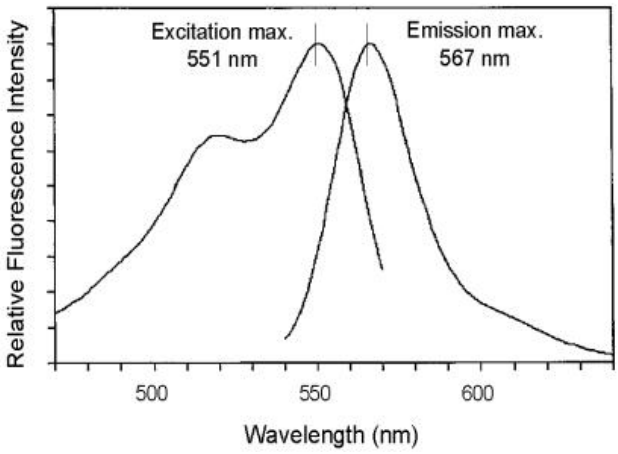
Storage

1. The PKH26 ethanolic dye solution 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.

2. Diluent C may be stored at room temperature or refrigerated.

If refrigerated, bring to room temperature before preparing cell and dye suspensions for labeling. 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.



Assay Procedure

A. General Cell Membrane Labeling

Lipophilic dyes bind to the cell membrane for labeling. The staining intensity is a function of the dye concentration and cell concentration, independent of permeability. Therefore, ensuring the appropriate amount of dye is crucial to avoid over-labeling, which can lead to loss of cell membrane integrity or reduced cell viability.

The following procedure can be used for labeling cells in vitro and in vivo, including stem cells, lymphocytes, monocytes, endothelial cells, neurons, or any other type of cells. The labeling process for in vivo cells may require certain modifications, such as staining of platelets or selective labeling of phagocytic cells.

In the following staining process, the cell concentration and dye concentration represent the initial concentrations used in the procedure. These concentrations have been proven to be suitable for various cell types. Users should determine the optimal dye concentration and cell concentration based on the experimental purpose by evaluating post-staining cell viability (e.g., PI staining), fluorescence intensity, staining uniformity, and whether it affects the functionality of the cells under investigation.

Note 1: During the PKH26 staining process, azides or metabolically toxic substances should not be present.

Note 2: Although adherent cells can also be stained, better staining uniformity is achieved with individual suspended cells. Therefore, for adherent cells, it is recommended to digest them into single suspended cells using a protease (trypsin/EDTA) for better staining results.

The following procedure has a final volume of 2 mL, a PKH26 concentration of 2×10^{-6} M, and a cell concentration of 1×10^{7} cells/mL. All the following steps are performed at room 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 1) prior to resuspension in Diluent C for labeling (step 4).

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

Note: The PKH26 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.

4. Prepare a 2x Cell Suspension by adding 1 mL of Diluent C to the cell pellet and resuspend with gentle pipetting to ensure 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 2x Dye Solution ($4x10^{-6}$ M) in Diluent C by adding 4 µL of the PKH26 ethanolic dye solution 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 $2x10^{-6}$ M is desired, the most reproducible results will be obtained by diluting the PKH26 ethanolic dye solution provided in the kit with undenatured 100% ethanol to make an intermediate dye stock.

6. Rapidly add the 1 mL of 2x Cell Suspension (step 4) to 1 mL of 2x Dye Solution (step 5) and immediately mix the sample by pipetting. Final concentrations after mixing the indicated volumes will be $1x10^{7}$ cells/mL and $2x10^{-6}$ M PKH26.

Note: 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:

a. Do not add ethanolic PKH26 dye directly to the 2x Cell Suspension in Diluent C.

b. Mix equal volumes of 2x Cell Suspension (step 4) and 2x Dye Solution (step 5).

c. Adjust 2x cell and 2x 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 mixing 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 400x 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 400x 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 (see 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.

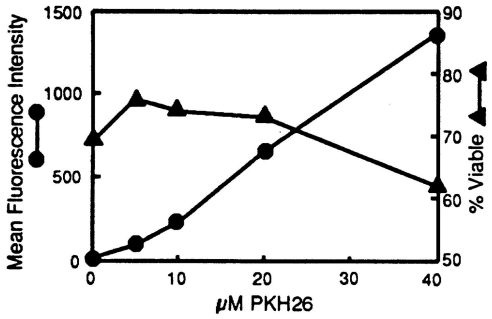


Fig. Staining Optimization for PKH26: MC-38 TIL cells were stained with the specified PKH26 concentration, resulting in a final cell concentration of 1×107 cells/mL. Cell viability (▲) was determined by FITC staining, and mean fluorescence intensity (●) was measured using flow cytometry. After labeling with 20 μM PKH26, the anti-tumor specificity and efficacy of TILs remained unchanged.

B. For Exosome / Extracellular Vesicle labeling

1. Begin with freshly isolated extracellular vesicle precipitates prepared using ultracentrifugation or microfiltration. Also, pre-cool the ultracentrifuge to 2-8°C.

2. Combine the precipitates from multiple tubes into one tube for each sample and measure the total volume.

3. For each sample, use diluent C from the PKH26 staining kit to bring the volume of the precipitate sample to 1 mL.

4. Identify the precipitate sample with the largest volume and add an equal volume of vesicle-free culture medium to a new ultracentrifuge tube. Bring the volume to 1 mL using diluent C.

5. Add 6 μL of PKH26 dye to each tube containing 1 mL of diluent C from steps 4-5.

6. Gently pipette up and down for 30 seconds. Let it stand for 5 minutes at room temperature.

7. Add 2 mL of 10% BSA (in PBS) for quenching. Bring the volume to 8.5 mL with serum-free medium.

8. Prepare a 0.971 M sucrose solution.

9. Slowly and carefully add 1.5 mL of the sucrose solution to the bottom of the tube, ensuring no vortex formation.

10. The extracellular vesicle-PKH26 solution will remain at the top of the sucrose cushion. Centrifuge at 190,000x g for 2 hours at 2-8°C.

Note: The extracellular vesicles will be located in the pellet, with most of the excess dye in the interface layer.

11. Carefully aspirate the culture medium and interface layer.

12. Gently resuspend the extracellular vesicle pellet in 1X PBS using a pipette.

13. Transfer to an Amicon filter column with a 10 kDa molecular weight cutoff (MWCO). Add 9 mL of PBS and 0.75 mL of

culture medium.

14. Centrifuge at 3000x g for 40 minutes to achieve a final retained volume of 0.5-1 mL.

15. Recover the concentrated solution from the Amicon column, store it in microcentrifuge tubes, and keep it on ice.

Analyze the fluorescence signal as soon as possible using the appropriate instrument.

Note: There is no standardized protocol for extracellular vesicle labeling. It is recommended to refer to other published articles for additional guidance and optimization.

Disclaimer

The reagent is only used in the field of scientific research, not suitable for clinical diagnosis or other purposes.