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# Product Information

**PKH26 Red Fluorescent Cell Linker Kit for Phagocytic Cell Labeling**

Catalog Number **PKH26PCL**

## TECHNICAL BULLETIN

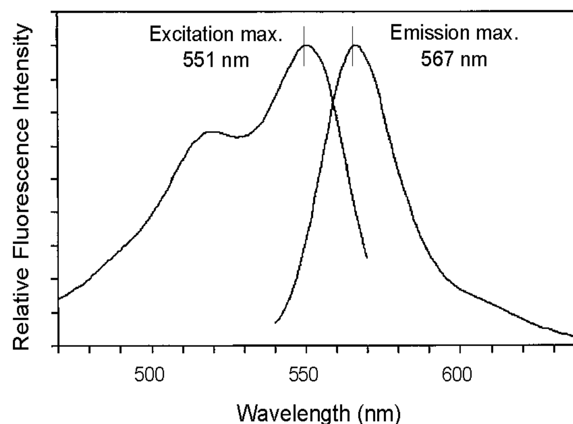
### Product Description

The PKH26PCL fluorescent cell linker kit selectively labels cells with phagocytic capabilities[1-21], including macrophages (MΦ),[1-17] neutrophils.[2,17,18] and microglia.[19] Labeling occurs through the ingestion of dye micro-aggregates formed when PKH26 is added to Diluent B. As previously reported for two green cell linker dyes PKH1 and PKH2,[22-24] dye aggregate formation significantly reduces staining efficiency for non-phagocytic cells, such as lymphocytes, while facilitating dye uptake by phagocytes. Labeled cells appear patchy or spotted because the dye is localized in phagocytic compartments of the cells.[22, 25] The ingested dye appears to be resistant to metabolic attack and has been found to remain with the cells for at least 21 days *in vivo.*[25]

PKH26 fluoresces in the yellow-orange region of the spectrum (**Figure 1**) and has been found to be useful for selective labeling of phagocytic cells both *in vitro* and *in vivo*. Intraperitoneal administration of PKH26 in Diluent B enables *in vivo* labeling of peritoneal macrophages,[3,5,7,9,12,16] and resident alveolar macrophages are readily labeled using either intravenous [2,4,6,8,15] or intranasal administration.[11] Intravenous injection of varying concentrations of PKH26 in Diluent B also allows labeling of a variety of mature circulating phagocytes[2,6,13,17,20] including neutrophils.[2, 17,18] In addition, cultured macrophages, microglia and other phagocytes of interest, e.g., hemocytes, may be stained *in vitro* using with PKH26PCL.[1,2,10,14,19,21]

The reagents in the PKH26PCL Cell Linker kit have been formulated to favor selective labeling of phagocytic cells over non-phagocytic cells, *e.g.*, lymphocytes, present in the population being stained. If labeling of both phagocytic and non-phagocytic cells is desired, one of the PKH26 cell linker kits configured for general membrane labeling (PKH26GL, MINI26) should be used rather than the PKH26PCL kit. Methods have also been reported for monitoring phagocytic cell function using PKH26GL-labeled targets such as bacteria[11], exosomes,[26] infected red blood cells,[27, 28] tumor cells[29, 30] and/or apoptotic neutrophils.[31]

**Figure 1.** PKH26 Excitation and Emission Spectra




### Components

- PKH26 dye stock (0.5 mL, 1 x $10^{-3}$ M in ethanol)
- Diluent B (6 x 10 mL)

### 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 PKH26 ethanolic dye solution (Catalog Number P9691) 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 B may be stored at room temperature or refrigerated. If refrigerated, bring to room temperature before use for *in vitro* or *in vivo* labeling. Diluent B is provided as a sterile low-endotoxin solution (<0.0625 EU/mL). Because it does not contain any preservatives or antibiotics, it should be kept sterile. **Do not store dye in Diluent B.**

**Equipment and Reagents Required but Not Provided for *In Vivo* Labeling of Phagocytic Cells**
Materials required for labeling peritoneal macrophages (Steps 1-7 of Procedure section):

- Mice or other animal species whose phagocytic cells are to be labeled.
- Absolute ethanol.
- 20 cc sterile syringe with needle.
- Sterile 1 cc syringes with 27 gauge 1" needles (1/animal, plus 1 extra).
- Bunsen burner or alcohol burner.
- Sterile gauze squares.
- 70% Ethanol in wash bottle.

Materials required for harvesting labeled peritoneal macrophages (Steps 8-14 of Procedure section):

- 70% Ethanol in wash bottle
- Forceps, surgical scissors and peg board.
- 5cc syringe with 20 or 22 gauge needle
- Dulbecco's phosphate buffered saline (PBS), ($Ca^{++}$ and $Mg^{++}$ free).
- 15 cc polypropylene centrifuge tubes
- Ice bucket with ice
- Additional materials for immunofluorescent staining as determined by the user.
- Instrument for analysis of fluorescence (fluorometer, fluorescence microscope, flow cytometer, or fluorescence image analysis instrumentation).

**Procedure for *in vivo* Labeling of Resident Peritoneal Macrophages**
This procedure has been optimized for labeling the resident peritoneal MΦ of Balb/c mice, approximately 20 gm body weight. The peritoneum of naive Balb/c mice (housed under virus free conditions) contains 20-30% MΦ.[22] Labeling conditions, including dye concentration and volume to be injected i.p., may need to be modified for other mouse strains which are larger or have more peritoneal MΦ.

1. Remove 0.1 ml PKH26 dye stock ($1 \times 10^{-3}$ M) from kit and mix thoroughly with 0.90 ml absolute ethanol to make a 100 μM working stock dye solution. Dye stock should be kept tightly capped when not in immediate use to prevent evaporation.
2. Remove the metal tab of one diluent bottle. Because the diluent bottles have been overfilled to assure at least a 10 ml volume, withdraw the contents of the bottle using a 20 cc syringe with needle, expel the overfill volume and reinject 10 ml back into the vial.
3. Remove 0.1-0.2 ml of the working stock (100 μM) with a 1 cc syringe. Inject 0.05 ml of the dye into the diluent bottle. If the needle will not penetrate the rubber stopper, the stopper may be removed. However, it is essential that the contents of the diluent vial remain sterile as any contamination will induce inflammation into the peritoneal cavity.
4. Replace the stopper, if necessary, and shake the bottle vigorously to mix the dye and diluent. Allow the solution to stand for at least 15 minutes.
5. Withdraw 0.5 ml of the diluted dye into a fresh sterile syringe for each animal to be injected. If possible, keep the rubber stopper on the diluent vial and flame the stopper before inserting each needle. Flame the needle again after withdrawing the dye, then flame the needle cap and place it over the needle.
6. Repeat step 5 for each syringe to be filled. Use a fresh syringe and needle for each animal. Shake the dye/diluent mixture before removing each sample for injection.
7. Inject the contents of each syringe i.p. Before each injection, swab the abdominal fur with a gauze pad soaked in alcohol. This procedure will minimize the incidence of inflammation from the i.p. injection.
8. Labeled peritoneal MΦ may be harvested by peritoneal lavage at any time from 2 hours to 21 days after the i.p. injection of PKH26 dye. To harvest the peritoneal cells, sacrifice the animal, e.g., by cervical dislocation, and wet the abdominal fur with 70% ethanol.
9. Make a small incision in the inguinal area cutting through the fur, but not through the dermis. Grasp the abdominal fur and retract the fur towards the shoulders. This procedure will expose the intact abdominal cavity.
10. Fill a 5 cc syringe with ice-cold PBS (larger volumes up to 10 ml may be preferred for larger mice), forcefully inject the PBS into the abdominal cavity, and withdraw the syringe. The injection site should be the inguinal fat pads. The abdominal fat will seal the injection site when the syringe is withdrawn.

11. Massage the abdominal cavity to loosen peritoneal cells adhered to the abdominal wall and viscera.
12. Insert the needle into the abdominal cavity, about midline, and gently withdraw the lavage fluid. With some experience, the user will be able to collect 90% of the injected fluid.
13. Place the contents of the syringe into a 15 cc polypropylene tube. The tube should be kept on ice to minimize adherence and/or clumping of the MΦ.
14. Centrifuge the peritoneal cells for 5 minutes at 350 x g, at 4 °C. The cells can then be resuspended and washed in saline or immuno-fluorescence wash buffer (for immunofluorescent staining) or media (for functional assays).

The procedure described here is designed to selectively label resident peritoneal MΦ of mice. The resident MΦ present in the peritoneum at the time of injection take up PKH26 and can thus be distinguished from subsequently recruited MΦ, which are not labeled. This procedure can be adapted to label other phagocytic cells, *e.g*. neutrophils,[2, 17,18] resident alveolar MΦ[1-17], dendritic cells,[3] microglia[19] or primitive phagocytes, [20,21] either *in vivo* or *in vitro*.

**Critical Aspects of Phagocytic Cell Labeling**

1. This procedure is designed to selectively label resident MΦ. If monocytes/ MΦ are recruited to the peritoneum as the result of inflammation induced by the *i.p*. injection, they will probably not be labeled by the dye. The peritoneal cavity is very sensitive to inflammation and the procedure described above utilizes several precautions to minimize this inflammation. These include keeping the injection vial and syringes absolutely sterile, using a fresh disposable syringe and 27 gauge needle for each animal, and wiping the abdominal fur with alcohol before each injection.
2. In spite of the precautions described above, some injection induced inflammation has been routinely observed in as many as 50% of the mice. This percentage will decline with practice. Injection induced inflammation is detected, following immunofluorescent labeling and flow cytometric analysis, as two populations of MΦ (dye labeled and unlabeled), or one broad population of MΦ with variable stain intensity, or very few to no labeled MΦ in extreme inflammation.[25,32] At 24 hours after injection, animals with inflammation can be identified by elevated peritoneal cell number and elevated neutrophil counts compared to untreated animals.
3. Extra animals may be injected for each study and after immunofluorescence pattern analysis and differential cell counts of each animal, data from the animals with inflammation is not included.[33]
4. In the absence of inflammation, resident peritoneal MΦ exhibit elevated red fluorescence intensity compared with recruited MΦ, and can be distinguished from the latter for at least 21 days *in vivo*.[25]
5. Diluent B has been tested for lipopolysaccharide (LPS) content and has been found to contain less than 0.0625 EU/ml.
6. The staining conditions should be optimized for each species (and strain) of animal tested, as well as for type of phagocytic cell being labeled and route of administration. Relatively low staining concentrations (0.5 –2.5 µM) have typically been used for intraperitoneal staining of resident peritoneal MΦ in mice[3,7,9,16] whereas concentrations of 10 - 15 µM have been reported as optimal for intravenous labeling of neutrophils in sheep[18] or resident alveolar MΦ in rats.[2,4] Intravenous injection of higher concentrations (50 - 200 µM) has also been reported, [6,8,13] with the highest concentrations achieving labeling of mature MΦ throughout the body[8] while leaving circulating monocytes and their precursors in bone marrow unlabeled to allow discrimination between resident and recruited cells. [2,4]
7. The PKH26PCL staining kit can also be used to label phagocytic cells *in vitro*. [1,2,10,14,19,21] As with *in* vivo labeling, optimal dye concentration may vary with cell type and function of interest. Use of concentrations in the 2-20 µM range have often been found to give satisfactory results, particularly when the general membrane labeling procedure for PKH26GL is adapted for labeling phagocytic cells by substituting PCLDIL(Diluent B) for CGLDIL (Diluent C). For a copy of the PKH26GL labeling procedure, go to http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Bulletin/pkh26glbul.Par.0001.File.tmp/pkh26glbul.pdf.

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