Cytoplasmic Membrane Staining Kits

PromoKine

Instruction Manual

Product Name	Size	Catalog Number
Green-fluorescent Cytoplasmic Membrane Staining Kit	1 ml	PK-CA707-30021
Orange-fluorescent Cytoplasmic Membrane Staining Kit	1 ml	PK-CA707-30022
Red-fluorescent Cytoplasmic Membrane Staining Kit	1 ml	PK-CA707-30023
Blue-fluorescent Cytoplasmic Membrane Staining Kit	50 assays	PK-CA707-30024

Introduction

The carbocyanine dyes DiI, DiO, DiD and DiB label cytoplasmic membrane and intracellular membrane structures efficiently and permanently [1]. They have been used as tracers in cell–cell fusion [2,3], cellular adhesion [4,5], and migration [6] applications due to their properties of low cytotoxicity and high resistance to intercellular transfer. However, the lipophilic nature of these dyes posed an obstacle to uniform cellular labeling. Although structurally related PKH dyes have been developed and optimized for cell labeling, the procedure requires multiple steps and subjects cells to an iso-osmotic mannitol loading medium that can affect cell viability [8,9]. PromoCell's Fluorescent Cytoplasmic Membrane Staining Kits are ready-to-use dye delivery solutions that can be added directly to normal culture media to uniformly label suspended or attached culture cells. In addition, NeuroDiO, an improved version of DiO, further improves green fluorescent cytoplasmic membrane labeling. PromoCell also offers DiB, the first first blue fluorescent lipophilic carbocyanine dye. In addition, we also supply *NIR Membrane Dyes* as well as *Fixable Membrane Stains* (see our website for more information).
PromoCell's fluorescent *Cytoplasmic Membrane Staining Kits* include cytoplasmic membrane orange labeling (DiI), cytoplasmic membrane green labeling (NeuroDiO), cytoplasmic membrane red labeling (DiD), and cytoplasmic membrane blue labeling (DiB). They allow cell populations to be marked in distinctive fluorescent colors for identification after mixing. Double labeling can identify cells that have fused or formed stable clusters.

Kit Contents

Product Number	Product Name	Content
PK-CA707-30021	Green-fluorescent Cytoplasmic Membrane Staining Kit	1 ml NeuroDiO Cell Labeling Solution
PK-CA707-30022	Orange-fluorescent Cytoplasmic Membrane Staining Kit	1 ml DiI Cell Labeling Solution
PK-CA707-30023	Red-fluorescent Cytoplasmic Membrane Staining Kit	1 ml DiD Cell Labeling Solution
PK-CA707-30024	Blue-fluorescent Cytoplasmic Membrane Staining Kit	Reagent A: 0.25 ml DiB Cell Labeling Solution Reagent B: 0.25 ml DiB Loading Buffer

Storage and Stability

Store cell labeling solutions at 4°C and protected from light. If solutions appear cloudy or precipitation has occurred, warm the vials to 37°C and vortex periodically until the dye has fully dissolved (the solution may be slightly turbid). Use solutions only when they are clear. Centrifuge the vials before opening the caps and seal the vials quickly and tightly after each use to avoid evaporation. When stored properly, the kit components should remain stable for up to 12 months from date of receipt.

Experimental Procedures

Before starting cell labeling, we recommend reading the *General Notes* and *FAQs* below.

1. *Labeling of Cells in Suspension*

1.1. Suspend cells at a density of 1×10^6/ml in normal cell culture growth medium.

1.2. Add 5 µl of the supplied *Cell Labeling Solution* per 1 ml of cell suspension. Mix well by by low-speed vortexing or flicking the tube.

Blue-fluorescent Cytoplasmic Membrane Staining Kit (PK-CA707-30024): Add 10 µL of the *Working Labeling Solution* per 1 mL of cell suspension and mix well.
Preparation of *Working Labeling Solution*
DiB Loading Buffer may solidify into a gel during storage. This is normal and does not affect the product, but the buffer must be in liquid form before use. Heat the solidified gel to 50-60°C for 5-10 minutes and vortex periodically until it has formed a clear liquid. When warming the vials in a water bath, make sure to completely submerge the upper conical portion of the vial that contains the liquid.
Prepare a 1:1 mixture of *DiB Cell Labeling Solution* (Reagent A) and *DiB Loading Buffer* (Reagent B) in a clean tube: Mix 5 µl of Reagent A with 5 µl of Reagent B per ml of staining medium required; Pipette the mixture up and down to mix thoroughly; this is your *Working Labeling Solution*. (Note: *Working Labeling Solution* should be prepared just before use). Add 10 µl of this *Working Labeling Solution* per 1 ml of cell suspension or staining medium as described below. Mix well by flicking the tube.

1.3. Incubate cells for 20 minutes at 37°C in the dark. The optimal incubation time will vary depending on cell type. Start by incubating for 20 minutes and optimize as needed to obtain uniform labeling.

1.4. Pellet the cells by centrifugation at 350 x g for 5 minutes.

1.5. Remove the supernatant and wash the cells by gently resuspending them in warm (37°C) medium.

1.6. Repeat the wash procedure (Steps 1.4 and 1.5) two more times.

1.7. Resuspend cells in medium and proceed with fluorescence observation.

2. *Labeling of Adherent Cells*

2.1. Culture adherent cells on sterile glass coverslips as either confluent or subconfluent monolayers.

2.2. Remove coverslips from growth medium and gently drain off or aspirate excess medium. Then place coverslips in a humidity chamber.

2.3. Prepare staining medium by adding 5 µl of the supplied *Cell Labeling Solution* to 1 ml of normal growth medium and mix well.

Blue-fluorescent Cytoplasmic Membrane Staining Kit (PK-CA707-30024): Prepare working solution as described above. Make staining medium by adding 10 µl of working solution to 1 ml of growth medium and mix well.

2.4. Add enough staining medium to completely cover the cells. Alternatively, *Cell Labeling Solution* (PK-CA707-30021-30023) or *Working Labeling Solution* (PK-CA707-30024) can be added directly to the cell culture and mixed well by shaking or swirling the plate (add 5 µl of *Cell Labeling Solution* or 10 µl of *Working Labeling Solution* [PK-CA707-30024] per ml of culture medium in the plate) but note that this may result in uneven labeling.
Blue-fluorescent Cytoplasmic Membrane Staining Kit (PK-CA707-30024): Remove medium from the cells and add enough staining medium to completely cover the cells. Alternatively, 10 µl of working labeling solution can be added directly to the cell culture and mixed well by shaking or swirling the plate, but this can cause uneven staining.

2.5. Incubate the cells at 37°C. The optimal incubation time will vary depending on the cell type. Start by incubating for 20 minutes and subsequently optimize as needed to obtain uniform labeling.

2.6. Remove the staining medium and wash the cells three times. For each wash cycle, cover the cells with fresh, warm (37°C) growth medium and incubate at 37°C for 5 minutes.

2.7. Add fresh medium to the cells and proceed with fluorescence observation.

Long Term Cell Staining

Lipophilic carbocyanine dyes are very stable and have been reported to stain live cells for weeks in culture (1) or *in vivo* (6) with minimal transfer between cells. Immediately after labeling cells, the dyes primarily stain the plasma membrane, even in fixed cells. However, dye localization in live cells changes over time. If cells are cultured after staining, the labeled membrane will be internalized, so staining will gradually change from cell surface to intracellular vesicles, usually becoming mostly intracellular after about 24 hours in commonly used immortalized cell lines.

Fixation After Staining

Live cells stained with carbocyanine dyes can be fixed with formaldehyde (PFA), but not methanol or other solvents. Staining can withstand permeabilization with 0.1% Triton® X-100 or 0.1% digitonin (10). However, permeabilization can alter the dye localization, resulting in increased intracellular staining. Alternatively, we have seen good preservation of plasma membrane staining when cells are fixed with formaldehyde, then permeabilized before staining with our Membrane Dyes (see Labeling Fixed Cells below).

Note: Do not use mounting medium with glycerol, which can cause altered staining and high background. Organic mounting media are also not suitable. We recommend imaging it directly in PBS (or other aqueous buffers). Coverslips should be mounted using PBS and sealed with a suitable coverslip sealant or nail polish. Stained samples can be stored in PBS at 4°C for several weeks or longer.

Also see as *Fixable Membrane Stains* and *Cell Surface Fixable Staining Kits* (at www.promocell.com). These are stains that covalently label cell membranes or the cell surface for truly fixable staining.

Labeling Fixed Cells

Note: Cells should be fixed with formaldehyde (PFA). Fixation with methanol or other solvents extracts lipids and results in poor staining.

1. Wash cells with PBS after fixation.
2. Optional: Permeabilize cells with 0.1% Triton® X-100 in PBS for 10 minutes at room temperature.
3. Note: We have found this condition to preserve plasma membrane staining better than digitonin or saponin permeabilization.
4. Wash the cells 3 times with PBS to remove all traces of detergent.
5. Optional: Perform staining with antibodies or other dyes. Do not use detergent in the buffers used for blocking, antibody dilution, or washing.
6. Prepare staining buffer by adding 5 µl of Cell Labeling Solution to 1 ml of PBS.
7. Remove the buffer from the cells and add the staining solution.
8. Incubate 10 minutes or longer at RT, in the dark.
9. Wash the cells 3 times with PBS.
10. Do not use mounting medium with glycerol, which can cause altered staining and high background. We recommend imaging in PBS.

General Notes:

a. It is recommended to optimize the staining procedure for each particular cell type. In some cases, it may be necessary to vary the staining volume and time.
b. If cells are returned to culture after staining, membrane internalization will occur, resulting in more intracellular staining and less surface staining over time.
c. Cells stained with carbocyanine dyes can be fixed with formaldehyde, but detergent permeabilization may adversely affect staining. Digitonin permeabilization (10 µg/ml -1 mg/ml) has been reported to be compatible with carbocyanine dye staining[10]. Avoid mounting medium containing glycerol.
d. Formaldehyde fixed cells can be stained using the same protocols above. Staining of fixed cells can be performed in PBS or other buffer at room temperature instead of culture medium at 37°C.
e. Also see frequently asked questions (FAQs) below.

Detection Configurations

Microscopy

Filter sets for detection of NeuroDiO, DiI, DiD and DiB are selected based on their spectral characteristics, as summarized in Table 1. Multiband filter sets are available for simultaneous detection of multiple tracers as follows:

- DiI and NeuroDiO = Omega XF52, Chroma 51004
- DiI and DiD = Omega XF92, Chroma 51007
- DiI, NeuroDiO and DiD = Omega XF93, Chroma 61005
- DiB, NeuroDiO and DiI = Chroma 61000V2

Omega® filters are supplied by Omega Optical, Inc. (www.omegafilters.com). Chroma filters are supplied by Chroma Technology Corp. (www.chroma.com).

Flow Cytometry

Cells labeled with DiI, NeuroDiO and DiD can be analyzed using the conventional FL2, FL1 and FL3 flow cytometer detection channels, respectively.

Cells labelled with DiB can be analysed using the UV laser line.

Table 1. Spectral characteristics of DiI, DiO, DiD and DiB.

Dye (Product Number)	Abs	Em	Optical Filters	
			Omega	Chroma
NeuroDiO (PK-CA707-30021)	484	501	XF23	31001 or 41001
DiI (PK-CA707-30022)	549	565	XF32	31002 or 41002
DiD (PK-CA707-30023)	644	665	XF47	31023 or 41008
DiB (PK-CA707-30024)	360	420	XF03	31000V2

Intended Use

For in vitro research use only. Not for diagnostic or therapeutic procedures.

References

1. J Cell Biol 103, 171 (1986); 2. J Cell Biol 135, 63 (1996); 3. Cytometry 21, 160 (1995); 4. J Biol Chem 273, 33354 (1998); 5. J Cell Biol 136, 1109 (1997); 6. Anticancer Res 18, 4181 (1998); 7. J Immunol Methods 156, 179 (1992); 8. Methods Cell Biol 33, 469 (1990); 9. US Patent 4,783,401; 10. J Neurosci Methods. 174, 71 (2008).

FAQs

Question	Answer
Do Cell Membrane Dyes included in the kit specifically stain the plasma membrane?	The Cytoplasmic Membrane Stains are lipophilic carbocyanine dyes. These dyes undergo an increase in fluorescence when they insert into lipid bilayers. Lipophilic carbocyanine dyes stably label the plasma membrane and other intracellular membranes of cells. They also can be used to stain artificial lipid bilayers. If cells are returned to culture after staining, membrane internalization will occur, resulting in more intracellular staining and less surface membrane staining over time.
How stable is membrane staining using these dyes? Are the dyes toxic to cells?	Lipophilic carbocyanine dyes have been used to stain neuronal cells in culture for several weeks, and in vivo for up to a year. The dyes do not appreciably affect cell viability, and do not readily transfer between cells with intact membranes, allowing cell migration and tracking studies in mixed populations. Stability of labeling may vary between cell types, depending on rates of membrane turnover or cell division.
Can cells be fixed after the membrane staining using these dyes? Can the dyes be used to stain cells or tissues after they are fixed?	Cells can be fixed with formaldehyde after labeling with the Cytoplasmic Membrane Stains. Lipophilic carbocyanine dyes like the dyes included in the kits have also been used to stain cells or tissues after formaldehyde fixation. Permeabilization of cells with detergents or solvents, or mounting medium containing glycerol may adversely affect staining. Permeabilization with digitonin (10 µg/ml to 1 mg/ml) has been reported to be compatible with lipophilic carbocyanine dye staining.

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