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Product Information

CellVue® Claret Far Red Fluorescent Cell Linker Kits
For General Cell Membrane Labeling

Catalog Numbers **MINCLARET and MIDCLARET**
Store at Room Temperature

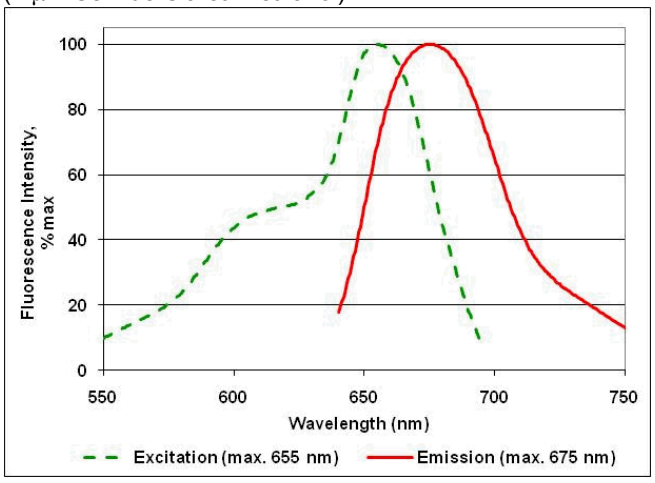
TECHNICAL BULLETIN

Product Description

The CellVue Claret Far Red Fluorescent Cell Linker Kits use proprietary membrane labeling technology to stably incorporate a far-red fluorescent dye with long aliphatic tails (CellVue Claret) into lipid regions of the cell membrane.[1,2] The labeling vehicle provided with the kit (Diluent C) is an aqueous solution designed to maintain cell viability, while maximizing dye solubility and staining efficiency.[3] Diluent C is iso-osmotic for mammalian cells and contains no detergents or organic solvents, but also lacks physiologic salts and buffers.[4] As with the PKH dyes, to which CellVue Claret is chemically related, 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.[2-6] However, CellVue Claret fluorescence is independent of pH within physiologic ranges and fluorescence intensity per cell is typically unaffected by pattern of dye localization.[5]

CellVue Claret fluoresces in the far red (see **Figure 1**). It has been reported to be useful for *in vitro* studies of lymphocyte proliferation and function,[1,7-9] *in vitro*[7] and *in vivo* cytotoxicity assays,[10] and for monitoring cell-cell membrane transfer.[11] It may also be useful as an alternate color for tracking tumor stem cell proliferation *in vitro*[12,13] or *in vivo*.[14]

Figure 1.
CellVue Claret Excitation and Emission Spectra
(1 µM CellVue Claret in ethanol)



Components

	CellVue Claret Dye* Catalog No.C0744	Diluent C Catalog No. G8278
MINCLARET	1 × 0.1 mL	1 × 10 mL
MIDCLARET	2 × 0.1 mL	6 × 10 mL

* 1×10^{-3} M in ethanol

The MINCLARET Kit is recommended for small or preliminary studies and the MIDCLARET Kit is recommended for *in vitro* proliferation or cytotoxicity 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
This product is 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 CellVue Claret ethanolic dye solution (Catalog Number C0744) may be stored at room temperature or refrigerated. To prevent increases in the 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, it should be warmed slightly in a 37 °C water bath, and sonicated or vortexed to redissolve the crystals.

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..

Procedure
A. General Cell Membrane Labeling [2-5,7]
Labeling occurs by partitioning of the lipophilic dye into cell membranes. Labeling intensity is a function of both dye concentration and cell concentration, and labeling 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, or any other cell type where partitioning of dye into lipid regions of the cell membrane is desired. [2-15] Modified procedures may be required for *in vivo* labeling[16] and for labeling platelets, or liposomes, or phagocytic cells.[2,17,18]

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 likely if the general cell 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.[4,7,15]

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

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 CellVue Claret 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. Therefore, 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: Do not add ethanolic CellVue Claret dye solution 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. 7 for suggested methods.

4. Prepare a 2× Cell Suspension by adding 1 mL of Diluent C (Catalog Number G8278) 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 CellVue Claret ethanolic dye solution (Catalog # C0744) 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 of $<2 \times 10^{-6}$ M is to be used, the most reproducible results will be obtained by diluting the CellVue Claret 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 by pipetting. Do not mix by "racking" or vortexing. Final concentrations after mixing the indicated volumes will be 1×10^7 cells/mL and 2×10^{-6} M of CellVue Claret.

Note: Because staining is nearly instantaneous, **rapid and homogeneous dispersion of cells in dye is essential for bright, uniform, reproducible labeling**. The following measures have been found to aid in optimizing results:[7]

a. Do not add ethanolic CellVue Claret dye directly to the 2× cell suspension in Diluent C.
b. Mix equal volumes of 2× cell suspension (step 4) and 2× Dye Working 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 adding and admixing cells 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 reagent**. Increase volume to 10 mL if complete medium is used instead of serum.

Note 2: Do not stop by adding Diluent C or centrifuge 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 aspirate supernatant, being careful not to remove any cells. Resuspend cell pellet in 10 mL of complete medium, transfer to a fresh sterile conical polypropylene tube, centrifuge at 400 × g for 5 minutes at 20-25 °C, and wash the cell pellet 2x more 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.

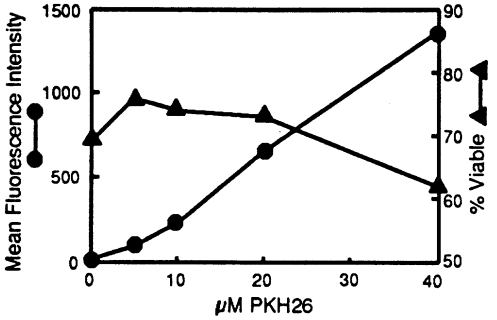
10. After the final wash, resuspend the cell pellet in 10 mL of complete medium for assessment of cell recovery, cell viability and fluorescence intensity (**Figure 2**). Centrifuge and resuspend to desired final concentration of viable cells.

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 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.[4,7]

Note 3: When CellVue Claret stained cells are to be viewed by epifluorescence microscopy, insure that appropriate filters are in place, *e.g.*, similar to those used for Cy® 5 or Cy5.5. Fluorescence will be most efficiently visualized using a photomultiplier or CCD camera, since the human eye has poor sensitivity at wavelengths >600 nm and the emission maximum for CellVue Claret is 675 nm.

Figure 2. Staining Optimization for CellVue Claret



CellVue Claret staining concentration may be optimized using a method similar to that described for PKH26.[15] 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 from flow cytometric histograms. Anti-tumor TIL specificity and potency *in vitro* and *in vivo* was confirmed to be unaltered by labeling with 20 µM PKH26.[15] *Reprinted with permission from the American Association for Cancer Research. Originally appeared in Cancer Research, **53**, 2360 (1993).*

Histology

PKH26 labeled adipocytes have been successfully identified in tissues subjected to standard paraffin embedding and sectioning,[19] but such methods risk loss of intensity because they use organic solvents in which membrane lipids and lipophilic dyes may be partially or completely soluble.[4,5]

Histologic studies of tissues containing cells labeled with lipophilic membrane dyes have typically been carried out on serial frozen sections[15] or sections prepared after fixation in neutral buffered formalin prepared from 4% paraformaldehyde.[20-21] 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.[15]

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 CellVue Claret.

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., Cy5 or Cy5.5 for CellVue Claret.

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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