Miltenyi Biotec

Fixation and Dead Cell Discrimination Kit

Order No. 130-091-163

Index

1. Description

Components	**200 µL Dead Cell Discriminator** **500 µL Discriminator Stop Reagent** **25 mL Fix Solution**
Size	Up to 100 tests with 10^7 cells
Product format	The Dead Cell Discriminator is supplied as a solution containing 0.05% sodium azide. The Discriminator Stop Reagent is supplied in a suspension containing 0.05% sodium azide. The Fix Solution contains 3.75% formaldehyde (EU Hazard Classification Xn harmful; R40/20/21/22-43).
Storage	Store protected from light at 4–8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 Principle of the Fixation and Dead Cell Discrimination Kit

The Fixation and Dead Cell Discrimination Kit is used to distinguish previously viable and dead cells in fixed samples, by flow-cytometric analysis. The Dead Cell Discriminator is a membrane-impermeant fluorescence dye, which infiltrates selectively, into dead cells because of their damaged membranes. In the cell, it binds to nucleic acids of the respective cells. The samples are then incubated under a 60W light bulb for a short time. Due to exposure to visible light, the binding becomes covalent and thus irreversible. After washing, the cells can be fixed. The final addition of Discriminator Stop Reagent allows optimal dead cell discrimination even after prolonged storage (up to 24 hours) of the fixed cells at 4–8 °C. The Dead Cell Discriminator is excited by a laser emitting light at 488 nm and has a fluorescence emission maximum at 625 nm. The fluorescence signal is very similar to that of propidium iodide, and accordingly does not need fluorescent compensation for flow-cytometric analysis.

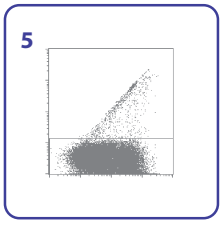
Dead Cell Discriminator and fluorescence-conjugated staining antibodies are added to the sample.

Cells are incubated for 10 minutes under a light source (photolabeling of dead cells).

(Optional) When recommended by the manufacturer, light-sensitive staining reagents can subsequently be added to the sample and incubated in the dark.

Cells are washed.

Fix Solution and Discriminator Stop Reagent are added to the sample.



Cells are analyzed by flow cytometry.

Fixation reduces sample hazard and allows storage for up to 24 hours prior to flow-cytometric acquisition.

Sensitivity of analysis is significantly enhanced due to discrimination of previously dead cells.

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1.2 Background and product applications

The Fixation and Dead Cell Discrimination Kit enables to distinguish previously viable and dead cells in fixed samples. Non-specific background staining can be excluded from analysis, thus sensitivity of detection can be enhanced. Fixation of cells facilitates working with bio-hazardous samples and allows prolonged storage of the prepared samples up to 24 hours.
The Fixation and Dead Cell Discrimination Kit can be used for staining of nucleated dead cells of any species.

Examples of applications

- Highly sensitive flow-cytometric analysis, such as rare cell analysis, that requires fixation to reduce bio-hazard, e.g. in HIV-infected samples.
- Highly sensitive flow-cytometric analysis of samples, that require fixation for prolonged storage.
- Flow-cytometric rare cell analysis after MACS® enrichment, on samples that require fixation.

1.3 Reagent and instrument requirements

- Buffer (degassed): PBS (phosphate buffered saline) pH 7.2, supplemented with 0.5% BSA and 2 mM EDTA. Keep buffer cold (4–8 °C).

  ▲ **Note:** EDTA can be replaced by other supplements such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as human serum albumin, human serum or fetal calf serum. Buffers or media containing Ca^{2+} or Mg^{2+} are not recommended for use.

- Fluorochrome-conjugated antibodies
- 60W light bulb
- Ice
- Closable transparent test tubes

Additional requirements for magnetic cell separation (see protocol 2.3)

- MACS® MicroBeads of choice
- MACS Columns and MACS Separators:

Column	max. number of labeled cells	max. number of total cells	Separator
Positive selection			
MS	10^7	$2x10^8$	MiniMACS, OctoMACS, VarioMACS, SuperMACS
LS	10^8	$2x10^9$	MidiMACS, QuadroMACS, VarioMACS, SuperMACS
XS	10^9	$2x10^{10}$	SuperMACS
autoMACS	$2x10^8$	$4x10^9$	autoMACS

▲ **Note:** Column adapters are required to insert certain columns into VarioMACS or SuperMACS. For details, see MACS Separator data sheets.

- (Optional) Pre-Separation Filter (# 130-041-407)

2. Protocol

2.1 Sample preparation

When working with anticoagulated peripheral blood or buffy coat, PBMC should be isolated by density gradient centrifugation (see "General Protocols" in the User Manuals or visit www.miltenyibiotec.com).

▲ **Note:** Remove platelets after density gradient separation: resuspend cell pellet in buffer and centrifuge at 200xg for 10–15 minutes at 20 °C. Carefully remove supernatant. Repeat washing step and carefully remove supernatant.

When working with tissues, prepare a single-cell suspension by a standard preparation method (see "General Protocols" in the User Manuals or visit www.miltenyibiotec.com).

2.2 Dead cell staining and fixation of fluorescently labeled cells

▲ Volumes for fluorescent labeling given below are for up to 10^7 total cells. When working with fewer than 10^7 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes, accordingly (e.g. for $2x10^7$ total cells, use twice the volume of all indicated reagent volumes and total volumes).

1. Resuspend up to 10^7 total cells in 100 µL of buffer.
2. Add antibodies for fluorescent staining.

   ▲ **Note:** When recommended by the manufacturer, light-sensitive fluorochrome-conjugated antibodies should be added after photolabeling of the Dead Cell Discriminator (see steps 4. and 5.).
3. Add **2 µL Dead Cell Discriminator** per 10^7 cells.
4. Mix well and incubate in a horizontal position on ice under a 60W light bulb (distance 3–5 cm) for 10 minutes.
5. (Optional) When recommended by the manufacturer, add light-sensitive fluorochrome-conjugated antibodies and incubate further in the dark.
6. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300xg for 10 minutes. Pipette off supernatant completely.
7. Resuspend cell pellet in 300 µL of buffer.
8. Add **150 µL Fix Solution** and **5 µL Discriminator Stop Reagent.** Mix well.
9. Proceed to flow-cytometric analysis (see example 3.1). Samples can be stored up to 24 hours prior to analysis.

2.3 Dead cell staining and fixation of magnetically enriched cells

▲ Work fast, keep the cells cold, and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labeling.

▲ Volumes for fluorescent and magnetic labeling given below are for up to 10^7 total cells. When working with fewer than 10^7 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for $2x10^7$ total cells, use twice the volume of all indicated reagent volumes and total volumes).

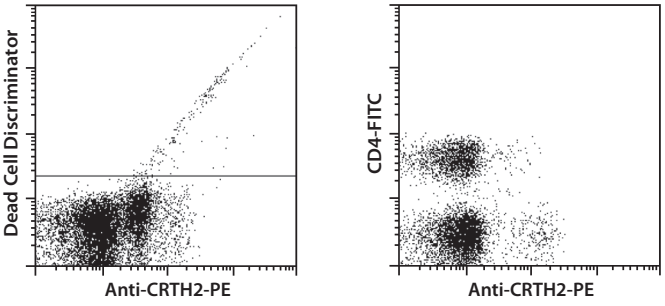
▲ For optimal performance it is important to obtain a single cell suspension before magnetic separation. Pass cells through 30 µm nylon mesh (Pre-Separation Filter # 130-041-407) to remove cell clumps which may clog the column.

1. Resuspend cell pellet in 80 µL of buffer per 10^7 total cells.
2. Add 20 µL of MACS® MicroBeads of choice per 10^7 total cells. Mix well and incubate for 15 minutes at 4–8 °C.
   ▲ **Note:** Working on ice may require increased incubation times. Higher temperatures and/or longer incubation times lead to non-specific cell labeling.
3. Add antibodies for fluorescent staining.
   ▲ **Note:** When recommended by the manufacturer, light-sensitive fluorochrome-conjugated antibodies should be added after photolabeling of the Dead Cell Discriminator (see steps 5. and 6.).
4. Add **2 µL Dead Cell Discriminator** per 10^7 cells.
5. Mix well and incubate in a horizontal position on ice under a 60W light bulb (distance 3–5 cm) for 10 minutes.
6. (Optional) add light-sensitive staining reagents and incubate further in the dark.
7. Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at 300xg for 10 minutes. Pipette off supernatant completely.
8. Resuspend cell pellet in 500 µL of buffer per up to 10^8 cells.
9. (Optional) Take an aliquot of the fraction before enrichment for flow-cytometric analysis.
10. Proceed to magnetic separation according to the datasheet of the respective MACS® MicroBeads.
11. (Optional) Take aliquots of the positive and/or negative cell fraction after magnetic separation for flow-cytometric analysis.
12. Centrifuge fractions of interest at 300xg for 10 minutes. Pipette off supernatant completely.
13. Resuspend cell pellets in 300 µL of buffer, respectively.
14. Add **150 µL Fix Solution** and **5 µL Discriminator Stop Reagent** to each fraction. Mix well.
15. Proceed to flow-cytometric analysis (see example 3.2). Samples can be stored up to 24 hours prior to analysis.

3. Examples

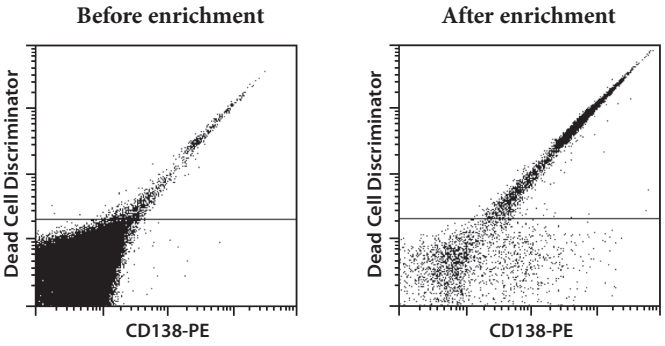
3.1 Dead cell discrimination after fixation of fluorescently labeled cells

PBMCs were stained with Anti-CRTH2-PE (# 130-091-238), CD4-FITC (# 130-080-501) and Dead Cell Discriminator. The sample was exposed to visible light. Cells were washed and fixed, Discriminator Stop Reagent was added to the sample. Lymphocytes were gated according to scatter properties by flow-cytometric analysis. Previously dead cells were excluded, in a FL2 (PE) versus FL3 (Dead Cell Discriminator) plot, by gating on the FL3-negative cells. CRTH2-expression on previously viable, fixed $CD4^+$ lymphocytes is shown.



3.2 Dead cell discrimination after fixation of magnetically enriched cells

PBMCs were labeled with CD138 MicroBeads (# 130-051-301), and stained with CD138-PE (# 130-081-301) and Dead Cell Discriminator. After magnetic separation using an MS Column in a MiniMACS™ Separator, cells were washed, fixed, and Discriminator Stop Reagent was added to the sample. Previously dead cells were excluded by flow-cytometric analysis, in a FL2 (PE) versus FL3 (Dead Cell Discriminator) plot, by gating on the FL3-negative cells.



Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com/local to find your nearest Miltenyi Biotec contact.

Warnings

Dead Cell Discriminator contains a potent mutagenic substance. Use appropriate protective equipment and methods to clean up spilled substances promptly. Collect and dispose of all waste properly.

Fix Solution contains formaldehyde and should be disposed of properly.
EU Hazard Classification for Inside Fix: Xn harmful;
R40/20/21/22: possibly irreversibly harmful if inhaled, swallowed or comes in contact with skin.
R43: sensitization through skin contact possible.

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