



Miltenyi Biotec

# Intracellular staining of eukaryotic cells

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## 1. Description

### Components

**Inside Fix**  
(containing 3.7% formaldehyde)  
(EU Hazard Classification:  
Xn harmful; R40/20/21/22-43)

**Inside Perm**  
(containing a detergent)

### Storage

Store protected from light at 4°C.

# Inside Stain Kit

25 ml Inside Fix,  
2x55 ml Inside Perm  
For up to 50 tests

Order No. 130-090-477

## 1.1 Principle of the intracellular staining

The Inside Stain Kit has been developed for intracellular staining of cells in suspension or of cells magnetically immobilized on a MACS® Separation Column.

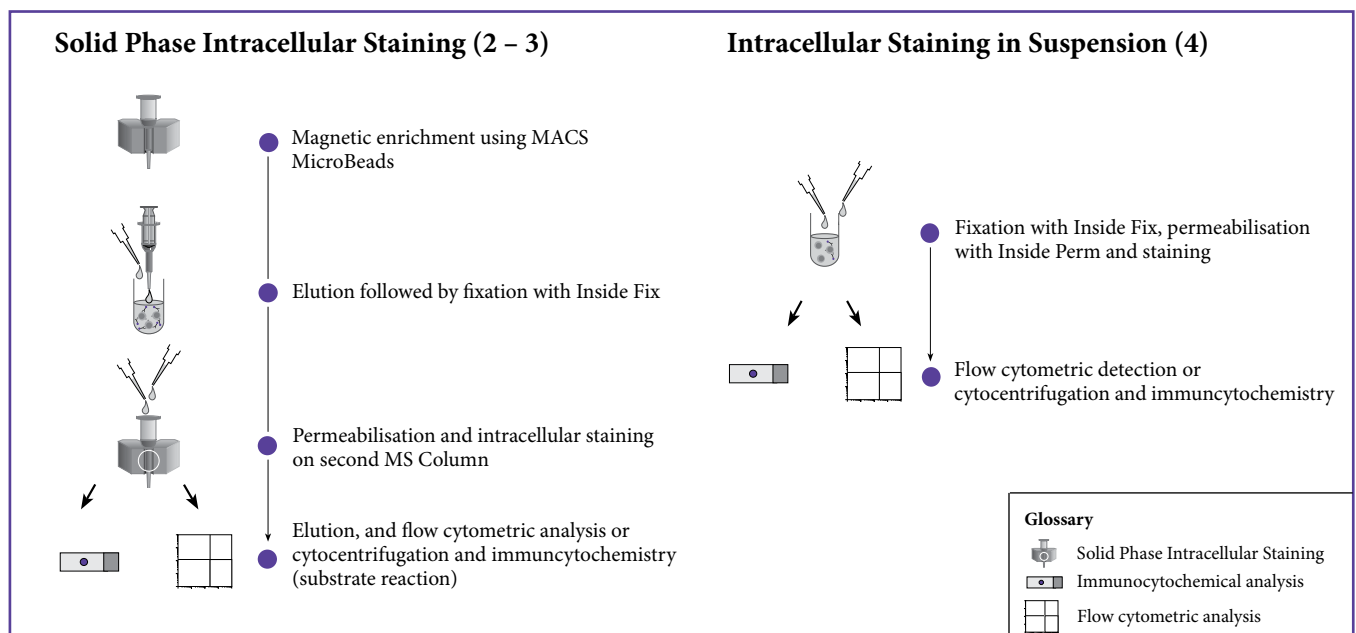
For staining of magnetically immobilized cells ("Solid Phase Intracellular Staining") using the Inside Stain Kit, the magnetically labeled cells are eluted from a MACS Column, fixed in suspension by adding Inside Fix and applied onto a second MACS Column. Here the cells are permeabilized by adding Inside Perm and then stained for intracellular antigens. The stained cells are eluted from the column for subsequent analysis by immunocytochemistry or flow cytometry.

In comparison to other cell staining techniques cell losses are minimized by avoiding centrifugation steps.

## 1.2 Applications

Typical applications for the Inside Stain Kit are:

- ▲ Immunocytochemical, fluorescence microscopic or flow cytometric detection of (enriched) carcinoma cells using Anti-Cytokeratin (FITC or AP-conjugated) antibodies.
- ▲ Immunocytochemical detection of enriched melanoma cells, based on expression of intracellular antigens, e.g. Melan A.
- ▲ Analysis of clonality of enriched B-Lymphoma or Myeloma cells by staining for intracellular immunoglobulin light chains.
- ▲ Fluorescence microscopic or flow cytometric analysis of intracellular cytokine expression.



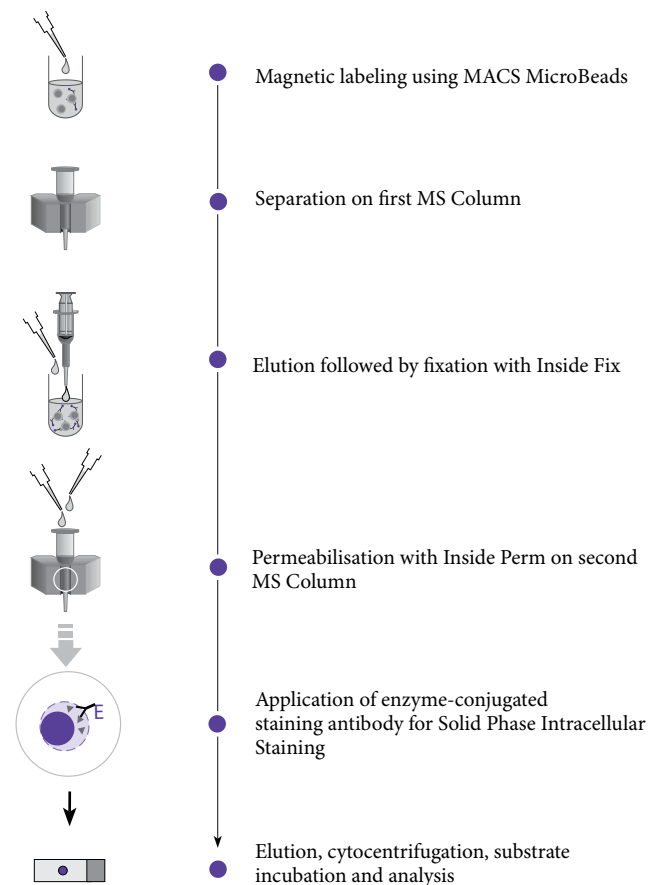
140-000-463304

## 2. Solid Phase Intracellular Staining using the Inside Stain Kit and immunocytochemical analysis

### 2.1 Reagent and instrument requirement

- Buffer: phosphate buffered saline pH 7.2, supplemented with 0.5 % bovine serum albumin and 2 mM EDTA.
- Staining antibodies for intracellular staining, e.g Anti-Cytokeratin Alkaline Phosphatase (# 130-090-462).
- Phosphate Buffered Saline pH 7.2 (PBS, e.g. Gibco Life Technologies, Paisley, UK).
- Substrate for Alkaline Phosphatase, e.g. SIGMA FAST™ Fast Red TR/Naphtol AS-MX tablets (Sigma-Aldrich, Deisenhofen, Germany).
- Meyer's hemalum solution (Merck, Darmstadt, Germany).
- Mounting medium, e.g. Faramount Aqueous Mounting Medium (DAKO, Hamburg, Germany).
- Cytocentrifuge, e.g. Hettich Universal (Andreas Hettich GmbH, Tuttlingen, Germany).
- Marking-pen, e.g. DAKO PEN (DAKO, Hamburg, Germany) or diamond pen.
- Silane coated slides, e.g. Histobond® (Marienfeld, Bad Mergentheim, Germany) and coverslips.
- MS Columns (# 130-042-201)
- Separators: MiniMACS, OctoMACS, VarioMACS, SuperMACS. Using VarioMACS or SuperMACS a MS Adapter is necessary.
- (Optional) Pre-Separation Filter (# 130-041-407)

### Protocol overview



### 2.2 Protocol for Solid Phase Intracellular Staining (immunocytochemical analysis)



#### Magnetic labeling

Label appropriate number of cells (up to  $2 \times 10^8$  total cells and up to  $10^7$  target cells) with MACS MicroBeads according to the instruction on the MACS MicroBead data sheet.

▲ **Note:** Detailed protocols for optimal magnetic labeling conditions are included with all MACS MicroBead data sheets.



#### Pre-enrichment and Solid Phase Intracellular Staining with enzyme-conjugated antibodies for immunocytochemical analysis

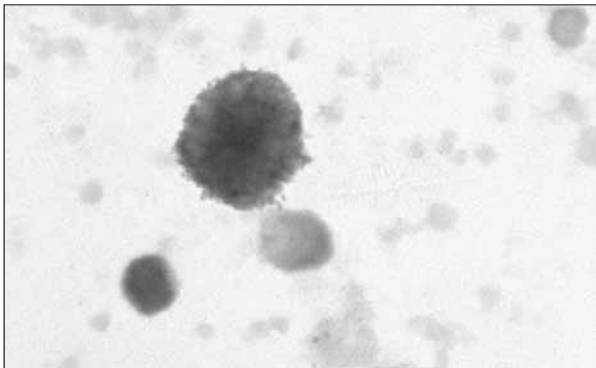
1. Choose a MS Column and place it in an appropriate MACS Separator (see "Column data sheets").
2. Prepare MS Column by rinsing with 500  $\mu$ l of degassed buffer (see "Column data sheets").
3. Pass cells through a 30  $\mu$ m nylon mesh (Pre-Separation Filter # 130-041-407) to remove clumps. Wet filters with degassed buffer before use.
4. Apply cell suspension in 0.5–1 ml of buffer onto the column (up to  $10^8$  cells per 500  $\mu$ l). Allow the negative cells to pass through. Wash with 3 x 500  $\mu$ l of buffer.
5. Remove the MS Column from separator, place column on a suitable collection tube, pipette 500  $\mu$ l of buffer onto the column. Firmly flush out retained cells using the plunger supplied with the column.
6. Add 500  $\mu$ l of Inside Fix to the positive cell fraction and incubate for 20 minutes at room temperature. **The final fixation volume is 1 ml.**
7. Place a new MS Column in an appropriate MACS Separator and prepare column by rinsing with 500  $\mu$ l of degassed buffer.
8. Apply the fixed cells onto the new MS Column, let cell suspension completely enter the column matrix and **immediately** wash with 500  $\mu$ l of buffer.
9. Permeabilize cells by applying 500  $\mu$ l of Inside Perm on the column.
10. Dilute enzyme-conjugated primary antibody in Inside Perm to appropriate titer (according to the manufacturer's instructions).
11. Apply 100  $\mu$ l of diluted antibody solution onto column and incubate for 10 minutes at room temperature.
12. (Optional for indirect stainings) Rinse column with 2 x 500  $\mu$ l of Inside Perm, apply 100  $\mu$ l of the secondary antibody diluted at appropriate titer in Inside Perm and incubate for 10 minutes at room temperature. For additional staining steps repeat steps 10.-12.
13. Wash column first with 500  $\mu$ l of Inside Perm followed by 500  $\mu$ l of PBS.
14. Remove MS Column from separator and flush out positive cells with 500  $\mu$ l of PBS using the plunger supplied with the column.
 

▲ **Note:** When using adhesion slides elute the cells in PBS without BSA or other proteins in order to enhance binding of the cells to the surface of the slides.

### 2.3 Immunocytochemical analysis after Solid Phase Intracellular Staining of magnetically enriched cells with enzyme-conjugated antibodies

1. After magnetic enrichment and Solid Phase Intracellular Staining spin cells onto a slide using a cytocentrifuge. Air-dry slide for 2-18 hours at room temperature.
2. Using a marking-pen (e.g. DAKO PEN) apply a hydrophobic line around the cell area on slide.
3. Wash slide for 2 minutes in PBS in a staining trough.
4. Prepare substrate solution for color reaction of the enzyme (e.g. Fast RedTR/Naphtol AS-MX substrate solution) according to manufacturer's instruction.
5. Add freshly prepared substrate solution to the cell area and incubate according to manufacturer's instruction.
6. Wash slide for 2 minutes in double-distilled water in a staining trough.
7. (Optional) Counterstain cells for 1 minute in filtered Meyers hemalum solution (diluted 1 to 2 in 100 mM TRIS-HCl, pH 8.2) in staining trough.
8. Wash slide for 2 minutes in double distilled water. Air-dry slide or mount with aqueous mounting medium.

### 2.4 Example for intracellular staining of enriched disseminated melanoma cells using the Inside Stain Kit



Enriched melanoma cells from peripheral blood of a patient with malignant melanoma stained according to the "Solid Phase Intracellular Staining" protocol by using the Inside Stain Kit with anti-Melan-A antibody, goat anti-mouse FITC and anti-FITC alkaline phosphatase. After elution from the column cells were spun down onto a slide by cytocentrifugation and incubated with a substrate for alkaline phosphatase.

## 3. Solid Phase Intracellular Staining using the Inside Stain Kit and flow cytometric analysis

### 3.1 Reagent and instrument requirement

- Buffer: phosphate buffered saline pH 7.2, supplemented with 0.5 % bovine serum albumin and 2 mM EDTA.
- Fluorochrome conjugated staining antibodies for intracellular staining, e.g. Anti-Cytokeratin-(CK3-6H5)-FITC (# 130-080-101).
- Columns and separators:

Column	max. number of labeled cells	max. number of total cells	Separator
MS	$10^7$	$2 \times 10^8$	MiniMACS, OctoMACS; VarioMACS, SuperMACS (with Column Adapter)

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

### 3.2 Protocol for Solid Phase Intracellular Staining (flow cytometric analysis)



#### Magnetic labeling

Label an appropriate number of cells (up to  $2 \times 10^8$  total cells and up to  $10^7$  target cells) with MACS MicroBeads according to the instruction on the MACS MicroBeads data sheet.

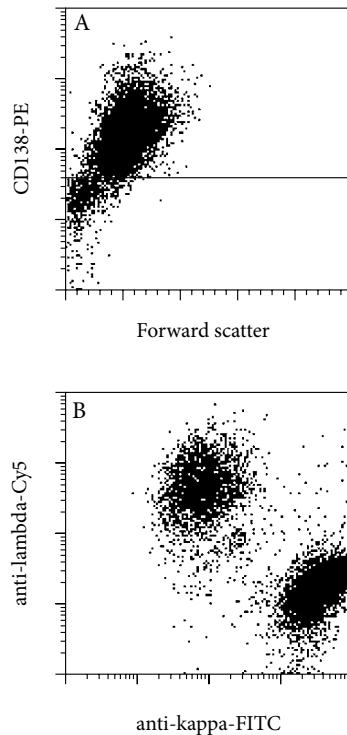
▲ **Note:** Detailed protocols for optimal magnetic labeling conditions are included with all MACS MicroBead data sheets.



#### Pre-enrichment, Solid Phase Intracellular Staining and flow cytometric detection

1. Choose a MS Column and place it in an appropriate MACS Separator (see "Column data sheets").
2. Prepare MS Column by rinsing with 500  $\mu$ l of degassed buffer (see "Column data sheets").
3. Pass cells through 30  $\mu$ m nylon mesh (Pre-Separation Filter # 130-041-407) to remove clumps. Wet filters with degassed buffer before use.
4. Apply cell suspension in 0.5–1 ml of buffer onto the MS Column (up to  $10^8$  cells per 500  $\mu$ l). Allow the negative cells to pass through. Wash with 3 x 500  $\mu$ l of buffer.
5. Remove the MS Column from separator, place column on a suitable collection tube, pipette 500  $\mu$ l of buffer onto the column and flush out positive cells using the plunger supplied with the column.
6. Add 500  $\mu$ l of Inside Fix to the positive fraction and incubate for 20 minutes at room temperature. **The final fixation volume is 1 ml.**
7. Place a new MS Column in an appropriate MACS Separator and prepare column by rinsing with 500  $\mu$ l of degassed buffer.
8. Apply the fixed positive cells onto the new MS Column, let cell suspension completely enter the column matrix and **immediately** wash with 500  $\mu$ l of buffer.
9. Permeabilize cells by washing the MS Column with 500  $\mu$ l of Inside Perm.
10. Dilute fluorochrome-conjugated primary antibody in Inside Perm at appropriate titer (according to the manufacturer's instructions).
11. Apply 100  $\mu$ l of diluted antibody solution onto column and incubate for 10 minutes at room temperature.
12. (Optional for indirect stainings) Rinse with 2 x 500  $\mu$ l of Inside Perm, apply 100  $\mu$ l of the secondary antibody diluted at appropriate titer in Inside Perm and incubate for 10 minutes at room temperature. For application of additional staining reagents steps 10.-12. may be repeated.
13. Wash column first with 500  $\mu$ l of Inside Perm followed by 500  $\mu$ l buffer.
14. Remove MS Column from separator and flush out positive cells with 500  $\mu$ l buffer using the plunger supplied with the column.
15. Cells eluted from the column are ready for immediate flow cytometric analysis.

### 3.3 Example for intracellular staining of enriched CD138<sup>+</sup> normal plasma cells using the Inside Stain Kit



CD138<sup>+</sup> plasma cells isolated from PBMC of a normal donor were stained with anti-kappa-FITC, CD138-PE and anti-lambda-Cy5 using the Inside Stain Kit and the "Solid Phase Intracellular Staining" protocol.

A. Gating of CD138<sup>+</sup> cells.

B. Staining on gated CD138<sup>+</sup> cells with anti-lambda-Cy5 versus anti-kappa-FITC.

## 4. Intracellular staining and flow cytometric analysis of cells in suspension using the Inside Stain Kit

### 4.1 Reagent and instrument requirement

- Buffer: phosphate buffered saline pH 7.2, supplemented with 0.5 % bovine serum albumin and 2 mM EDTA.
- Fluorochrome conjugated staining antibodies.



### 4.2 Protocol for intracellular staining in suspension and flow cytometric detection

1. Resuspend 10<sup>6</sup> PBMC in 250 µl of buffer and transfer into an 1.5 ml Eppendorf tube.
2. Add 250 µl of Inside Fix. The final fixation volume is 500 µl per 1 x 10<sup>6</sup> cells
3. Mix well and incubate for 20 min at room temperature.
4. Centrifuge cells for 5 min at 300 x g.
5. Remove supernatant carefully and resuspend pellet in 1 ml of buffer.
6. Centrifuge cells for 5 min at 300 x g and remove supernatant completely.
7. Dilute staining antibody e. g. Anti-Cytokeratin-(CK3-6H5)-FITC (# 130-080-101) in Inside Perm at appropriate titer (according to the manufacturer's instructions) to a final volume of 100 µl and resuspend cell pellet in diluted staining antibody.
8. Incubate for 10 minutes at room temperature.
9. Add 1 ml of Inside Perm.

10. Centrifuge cells for 5 min at 300 x g.
11. Remove supernatant carefully and resuspend pellet in 0.5 -1 ml of buffer.
12. Proceed to flow cytometric analysis.

## 5. Important notes

- ▲ Intracellular staining and staining of cell surface antigens can be performed in parallel. Make sure that surface antigens are formaldehyde-resistant.
- ▲ For efficient permeabilisation upon intracellular staining dilute staining antibody in Inside Perm at least 1:5.
- ▲ EDTA in the buffer can be replaced by other supplements such as acid citrate dextrose (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as gelatine, human serum or fetal calf serum. Buffers or media containing Ca<sup>2+</sup> or Mg<sup>2+</sup> are not recommended for use.
- ▲ Higher temperatures and longer incubation times for staining and magnetic labeling may lead to unspecific cell labeling.
- ▲ Fluorescently labeled cells can also be analysed by fluorescence microscopy instead of flow cytometry.

## 6. References

1. Siewert, C; Herber, M; Hunzelmann, N; Fodstad, O; Miltenyi, S; Assenmacher, M; Schmitz, J (2000) Rapid enrichment and Detection of Melanoma Cells from Peripheral Blood Mononuclear Cells by a New Assay Combining Immunomagnetic Cell Sorting and Immunocytochemical Staining. Recent Results in Cancer Research 158: 51-60. [921]

### Warning

Inside Fix 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.

### Warranty

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