



Immunocytochemical and immunohistochemical staining

Anti-Cytokeratin-Alkaline Phosphatase human

Order no. 130-090-462

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1. Description

Clone	CK3-11D5 and CD3-3E4; isotypes: mouse IgG1 and IgG2a
Product format	100 µL of alkaline phosphatase-conjugated anti-human cytokeratin antibody cocktail. The cocktail consists of two mouse monoclonal antibodies recognizing epitopes of differing cytokeratins. The antibody cocktail is supplied in a solution containing stabilizer and 0.05% sodium azide.
Product size	For up to 50 tests.
Storage	Store protected from light at 4–8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 Background and product applications

MACS® Anti-Cytokeratin-Alkaline Phosphatase has been developed for the direct immunohistochemical and immunocytochemical staining of fixed normal epithelial cells or epithelium-derived tumor cells, respectively, according to their expression of cytokeratins. Cell staining can be performed with or without prior magnetic enrichment. Cytokeratins are typical intermediate filaments of the cytoskeleton of epithelial cells. Most malignant cells that have their origin in epithelial tissue express cytokeratin and can be recognized with anti-cytokeratin antibodies. MACS Anti-Cytokeratin-Alkaline Phosphatase is a cocktail of two cytokeratin-specific antibodies CK3-11D5 and CK3-3E4 that are directly conjugated to alkaline phosphatase. CK3-11D5 is a multicytokeratin-specific antibody and recognizes cytokeratins 7, 8, 18 and possibly 19, and blocks the binding of Cam5.2, an antibody known to be specific for cytokeratins 7 and 8. Whereas CK3-3E4 binds specifically to cytokeratin 8.

Examples of applications

- Detection of disseminated epithelial tumor cells in peripheral blood, bone marrow or lymphoid tissue by immunocytochemistry, with or without prior magnetic enrichment.
- Immunohistochemical staining of disseminated epithelial tumor cells in formalin-fixed and paraffin-embedded or in frozen sectioned human tissue, for example primary tumor tissues.
- Immunohistochemical staining of normal epithelial cells in frozen sectioned or formalin-fixed, paraffin-embedded human tissues

1.2 Reagent and instrument requirements

For immunohistochemistry of paraffin-embedded tissue sections

- Phosphate buffered saline (PBS)
- Tris-buffered saline (TBS): 150 mM NaCl, 50 mM Tris/HCl, pH 7.6
- Blocking buffer: TBS with 5% (w/v) milk powder
- Antibody dilution buffer: TBS with 1% (w/v) milk powder
- Roti®-Histol (xylol substitute) (Roth # 6640.1)
- Isopropanol
- Ethanol dilution series (96%, 80%, 70% (v/v))
- Deionized water
- 10× Target Retrieval Solution, pH 6.0 (DakoCytomation # S1699)
- 3% H₂O₂ solution
- Hydrophobic pen (e.g. DakoCytomation Pen, DakoCytomation # S2002)
- Silane-coated slides (e.g. Histobond®, Superior # 901236)
- Coverslips (e.g. Roth 24×40 mm # 1870.1)
- Hellendahl jars
- Humidified incubation chamber
- SIGMAFAST™ Fast Red TR/Naphtol AS-MX Tablets (Sigma # F4648)
- Methylene green (e.g. Sigma # M7766)
- Mounting medium (e.g. Fluoromount-G, Southern Biotech # 01001-1)

For immunocytochemistry of cytospin preparations

- MACS® Inside Stain Kit (# 130-090-477)
- PBS
- Hydrophobic pen (e.g. DakoCytomation Pen, DakoCytomation # S2002)
- Silane-coated slides (e.g. Histobond®, Superior # 901236)
- Coverslips (e.g. Roth 24×40 mm # 1870.1)
- Hellendahl jars
- Humidified incubation chamber

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- SIGMAFAST™ Fast Red TR/Naphtol AS-MX Tablets (Sigma # F4648)
- (Optional) Meyer's hemalum solution (Merck # 109249), diluted 1:2 in 100 mM TRIS-HCl, pH 8.2
- Mounting medium (e.g. Fluoromount-G, Southern Biotech # 01001-1)

For magnetic pre-enrichment, fixation, and permeabilization

- Buffer (degassed): Prepare a solution containing PBS (phosphate buffered saline) pH 7.2, 0.5% BSA (bovine serum albumin) and 2 mM EDTA by diluting MACS BSA Stock Solution (# 130-091-376) 1:20 with autoMACS™ Rinsing Solution (# 130-091-222). 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 Ca²⁺ or Mg²⁺ are not recommended for use.

- CD326 (EpCAM) MicroBeads or Anti-ErbB-2 MicroBeads (# 130-061-101 and # 130-090-482, respectively)
 - PBS
 - MS Columns (# 130-042-201)
 - Separator: MiniMACS™, OctoMACS™, VarioMACS™ or SuperMACS™
- ▲ **Note:** When using VarioMACS™ or SuperMACS™ Separators, an MS Adapter is also required.
- MACS Inside Stain Kit (# 130-090-477)
 - Cyto centrifuge system (e.g. Hettich)
 - Silane-coated slides (e.g. Histobond®, Superior # 901236)
 - Hydrophobic pen (e.g. DakoCytomation Pen, DakoCytomation # S2002)
 - SIGMAFAST™ Fast Red TR/Naphtol AS-MX Tablets (Sigma # F4648)
 - Mounting medium (e.g. Fluoromount-G, Southern Biotech # 01001-1)
 - (Optional) Pre-Separation Filter (# 130-041-407)
 - (Optional) Meyer's hemalum solution (Merck # 109249), diluted 1:2 in 100 mM Tris/HCl, pH 8.2

2. Protocols

2.1 Immunohistochemical staining of paraffin-embedded tissue sections

1. Prepare sections of Paraffin-embedded tissues of no more than 2 cm in diameter.
2. Deparaffinize and rehydrate the tissue section by serial immersion in Hellendahl jars containing the following:
 - 2 × 5 min Roti-Histol
 - 2 × 3 min isopropanol
 - 2 × 3 min 96% ethanol
 - 2 × 3 min 80% ethanol
 - 2 × 3 min 70% ethanol
 - 2 × 3 min deionized water
3. For antigen retrieval, dilute Target Retrieval Solution 1:10 in deionized water and heat to 89 °C. Incubate tissue sections for 10 min, then allow to cool to room temperature.

4. Wash slide 2 × 3 min in PBS in a Hellendahl jar.
5. Block endogenous peroxidase activity by immersing slide in 3% H₂O₂ solution for 10 min at room temperature.
6. Wash slide 2 × 3 min in PBS in a Hellendahl jar.
7. Dab slide dry around the section and encircle it with a hydrophobic pen.
8. Block non-specific binding of antibody by incubating slide at room temperature for 20 min in blocking buffer.
9. Drain off supernatant by tilting the slide. Do not wash the slide.
10. Pipette 150 µL of Anti-Cytokeratin-Alkaline Phosphatase antibody, diluted 1:50 in antibody dilution buffer, to each section and incubate slide for 1 hour at room temperature in a humidified chamber.
11. Wash slides 2 × 3 min in deionized water in a Hellendahl jar.
12. Prepare substrate solution according to the manufacturer's instructions, mixing thoroughly before use.
 - ▲ **Note:** Prepare immediately before use.
13. Apply 150 µL of freshly prepared substrate solution to each tissue section and incubate for 20 min at room temperature.
14. Wash slide 2 × 3 min in deionized water in a Hellendahl jar.
15. (Optional) counterstain cells for 1 min by immersion in filtered Meyer's hemalum solution in a Hellendahl jar.
16. Wash slides for 2 min in deionized water in a Hellendahl jar.
17. Apply aqueous mounting solution and coverslip.

2.2 Immunocytochemical detection of cytokeratin-positive cells from cytospin preparations

1. Transfer 0.5–1 × 10⁶ mononuclear cells resuspended in PBS onto silane coated slides using a cyto centrifuge. Air-dry slide for 2–18 hours at room temperature.
2. Using a hydrophobic pen, encircle the pelleted cells.
3. Wash cells for 2 min in PBS in a Hellendahl jar.
4. For fixation of cells, apply 300 µL of MACS Inside Fix to the cell spot and incubate for 10 min at room temperature.
5. Drain off supernatant by tilting the slide.
6. Wash slide 3 × 3 min in PBS in a Hellendahl jar. Drain off excess PBS.
7. Dilute the Anti-Cytokeratin-Alkaline Phosphatase 1:50 in MACS Inside Perm, preparing enough to apply 250 µL per cell spot.
8. Apply 250 µL antibody per cell spot and incubate for 45 min at room temperature.
9. Drain off antibody by tilting the slide.
10. Wash slide 3 × 3 min in PBS in a Hellendahl jar.
11. Prepare substrate solution according to the manufacturer's instructions, mixing thoroughly before use.
 - ▲ **Note:** Prepare immediately before use.
12. Apply 250 µL of freshly prepared substrate solution to each tissue section and incubate for 20 min at room temperature.

13. Wash slide for 2 min in deionized water in a Hellendahl jar.
14. (Optional) counterstain cells for 1 min by immersion in filtered Meyers hemalum solution in a Hellendahl jar.
15. Wash slide for 2 min in deionized water. Air-dry slide or mount with aqueous mounting medium and apply coverslip.

2.3 Magnetic pre-enrichment and Solid Phase Intracellular Staining

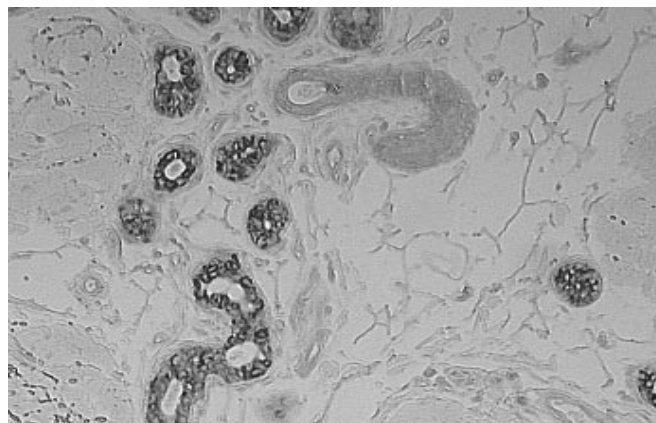
▲ Purification of tumor cells with MACS MicroBeads can be performed using either CD326 (EpCAM) MicroBeads or Anti-ErbB-2 MicroBeads, as listed above.

1. Label appropriate number of cells (up to 2×10^8 total cells or up to 10^7 target cells) with MACS MicroBeads, according to the respective datasheet.
2. Place an MS Column in the appropriate MACS Separator.

▲ **Note:** Detailed protocols for optimal magnetic labeling conditions are included with all MACS MicroBead data sheets.
3. Prepare MS Column by rinsing with 500 μ L of wash buffer.
4. (Optional) Pass cells through a 30 μ m nylon mesh (Pre-Separation Filter # 130-041-407) to remove clumps. Wet filters with wash buffer before use.
5. Apply cell suspension in 0.5–1 mL of wash buffer to the column (up to 10^8 cells per 500 μ L). Allow the negative (nonlabeled) cells to pass through. Wash with 3×500 μ L of wash buffer.
6. Remove the MS Column from the separator. Place column in a suitable collection tube, pipette 500 μ L of wash buffer onto the column. Firmly flush out retained cells using the plunger supplied with the column.
7. Add 500 μ L of Inside Fix to the positive cell fraction and incubate for 20 min at room temperature. The final fixation volume is 1 mL.
8. Place a new MS Column in the appropriate MACS Separator and prepare column by rinsing with 500 μ L of wash buffer.
9. Apply the fixed cells to the new MS Column, let cell suspension completely enter the column matrix and then **immediately** wash with 500 μ L of wash buffer.
10. Permeabilize cells by applying 500 μ L of Inside Perm to the column.
11. Dilute Anti-Cytokeratin-Alkaline Phosphatase 1:50 with Inside Perm.
12. Apply 100 μ L of diluted antibody solution to the column and incubate for 10 min at room temperature.
14. Wash column first with 500 μ L of Inside Perm followed by 500 μ L of PBS.
15. Remove MS Column from separator and flush out positive cells with 500 μ L of PBS using the plunger supplied.

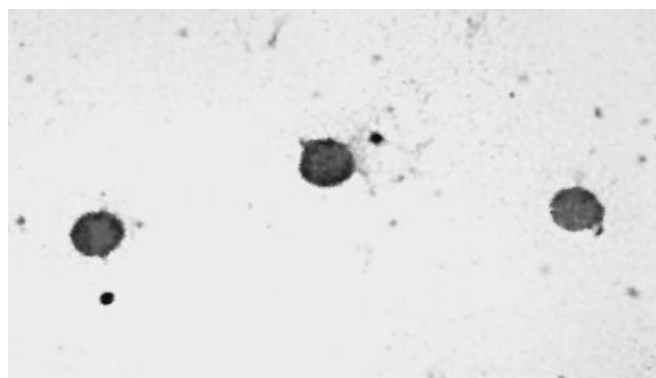
▲ **Note:** When using adhesive slides, elute the cells in PBS **without** BSA or other proteins in order to enhance binding of the cells to the surface of the slide.
16. Proceed with 2.2 Immunocytochemical detection of cytokeratin-positive cells from cytospin preparations, omitting steps 4–10.

3. Example of immunohistochemically detected cytokeratin-positive epithelial cells in skin



Epithelial cells (red) lining sweat glands in human skin are detected by immunohistochemical staining for cytokeratins in a 2 μ m paraffin-embedded section of a skin biopsy, using the Anti-Cytokeratin-Alkaline Phosphatase antibody cocktail and SIGMAFAST™ Fast Red TR/Naphtol AS-MX substrate.

4. Example of immunocytochemically detected cytokeratin-positive tumor cells in PBMCs



Cells of the carcinoma cell line SK-BR-3 were spiked into 5×10^7 PBMCs (peripheral blood mononuclear cells) of a healthy donor. The target cells were magnetically immobilized on a MACS Column and stained for cytokeratin reactivity by MACS Solid Phase Intracellular Staining using the MACS Inside Stain Kit, the Anti-Cytokeratin-Alkaline Phosphatase antibody cocktail and SIGMAFAST™ Fast Red TR/Naphtol AS-MX substrate.

For further information visit our website www.miltenyibiotec.com

Warnings

Reagents contain sodium azide. Under acidic conditions sodium azide yields hydrazoic acid, which is extremely toxic. Azide compounds should be diluted with running water before discarding. These precautions are recommended to avoid deposits in plumbing where explosive conditions may develop.

Warranty

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