

# MACS

## Cell Isolation Kit

## Carcinoma Cell Enrichment and Detection Kit

### Immunocytochemistry

For 1–3 x 10<sup>9</sup> total cells  
(up to 20 tests)

Order No. 130-060-301

### Overview

The Carcinoma Cell Enrichment Kit is a magnetic labeling system for the enrichment of disseminated epithelial tumor cells from peripheral blood, bone marrow or lymphoid tissue of patients with metastatic carcinomas. Most malignant cells which have their origin in epithelial tissue express cytokeratin and can be recognized by an anti-cytokeratin antibody.<sup>1</sup> With the Carcinoma Cell Enrichment and Detection Kit, disseminated epithelial tumor cells can rapidly and efficiently be enriched using MACS Cytokeratin MicroBeads. About a 10,000–fold enrichment, e.g. from a frequency of 1 tumor cell in 10<sup>7</sup> leukocytes to a frequency of 1 tumor cell per 10<sup>3</sup> leukocytes, makes detection and quantification of the tumor cells easy and reliable.

Enriched carcinoma cells can be detected with an anti-cytokeratin antibody. Immunocytochemical staining allows the specific detection of the tumor cells as well as their morphological analysis.

### Research Applications

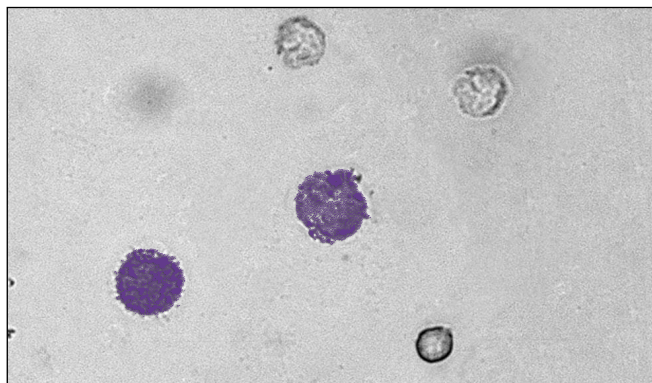
- ▲ Detection and quantification of disseminated carcinoma cells in peripheral blood, bone marrow or lymphoid tissue.
- ▲ Molecular characterization of circulating carcinoma cells from peripheral blood, bone marrow or lymphoid tissue of carcinoma patients.

### Enrichment and Detection Strategy

Enrichment of disseminated carcinoma cells from peripheral blood, bone marrow or lymphoid tissue is performed by positive selection of cytokeratin 7/8 expressing cells. For direct immunomagnetic labeling of intracellular cytokeratin 7/8, cells are permeabilized with MACS CellPerm Solution, fixed with MACS CellFix Solution and incubated with MACS Cytokeratin MicroBeads in MACS CellStain Solution. The magnetically labeled cells are enriched on a positive selection column in the magnetic field of a MACS separator.

For immunocytochemical detection of carcinoma cells in the MACS-enriched cell fraction, the cells are first incubated with FITC-conjugated anti-cytokeratin antibody and in a second step with anti-FITC antibody conjugated to alkaline phosphatase. These staining steps are performed in suspension before magnetic enrichment. After MACS enrichment cells of the magnetic fraction are spun on slides and incubated with alkaline phosphatase substrate.

### Cytokeratin Expressing Cells after Enrichment Using the Carcinoma Cell Enrichment Kit



Cytokeratin expressing cells from peripheral blood of a breast cancer patient were enriched using the Carcinoma Cell Enrichment Kit. Cells from the enriched fraction were immunocytochemically stained using a FITC-conjugated anti-cytokeratin antibody in combination with anti-FITC antibody-conjugated alkaline phosphatase and substrate.

### Components

- 2 x 50 ml 10xDilution Buffer.
- 50 ml MACS CellPerm Solution: contains detergent.
- 50 ml MACS CellFix Solution: contains formaldehyde.
- 50 ml 10xMACS CellStain Solution: contains detergent.
- 2 ml FcR Blocking Reagent: Human IgG.
- 2 ml MACS Cytokeratin MicroBeads: Colloidal super-paramagnetic MACS MicroBeads conjugated to a monoclonal anti-cytokeratin 7/8 antibody (Clone: CAM5.2,<sup>2</sup> Isotype: IgG2a).
- 1 ml Anti-Cytokeratin-FITC: FITC-conjugated anti-cytokeratin 7/8 antibody (Isotype: Mouse IgG2a).
- 100 µl Anti-FITC Alkaline Phosphatase: Alkaline Phosphatase conjugated to an anti-FITC antibody (Isotype: Mouse IgG1).
- 10 SIGMA FAST™ Fast Red TR/Naphthol AS-MX substrate tablets.
- 10 Tris/HCl tablets.

Reagents except for MACS CellFix Solution and tablets contain sodium azide.

### Storage

Store protected from light at 4°C. Do not freeze.

### Equipment Required

- Magnetic cell separator MiniMACS, VarioMACS or SuperMACS.
- MACS positive selection columns MS<sup>+</sup> or RS<sup>+</sup> plus RS<sup>+</sup> column adaptor.
- Pre-Column Separation Filters (Order No. 130-041-407).

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- Centrifuge, e.g. HETTICH Universal Centrifuge with HETTICH Cyto-System (Andreas Hettich GmbH, Tuttlingen, Germany).
- Marking-pen: DAKO PEN (DAKO, Hamburg, Germany) or diamond pen.
- Slides and coverslips.
- Staining throughs, type Hellendahl.
- Humidity chamber.

### Additional Reagents and Equipment Required

- Phosphate buffered saline (PBS), pH 7.2.
- 100 mM Tris-HCl, pH 8.2.
- MEYER'S hemalum solution (E. Merck, Darmstadt, Germany).
- Kaisers glycerol gelatin (E. Merck).

#### For bone marrow

- HEPES buffered cell culture medium (e.g. Iscove's modified Dulbecco medium [IMDM] from Gibco Life Technologies, Paisley, UK).
- Heparin (e.g. Liquimin from Hoffmann La Roche AG, Basel, Switzerland).
- DNase I (e.g. Boehringer Mannheim, Germany).

#### For cryopreserved material

- Dulbecco's PBS, containing 0.5 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub> (e.g. Gibco Life Technologies, Paisley, UK), pH 7.0- 7.2.
- DNase I (e.g. Boehringer Mannheim, Germany).
- BSA (e.g. Fluka, Buchs, Switzerland).
- Waterbath (40°C).
- **Cryo Dilution Buffer:** Add 0.25 g BSA and 1250–2500 U DNase to 25 ml of Ca<sup>2+</sup> and Mg<sup>2+</sup> containing PBS. About 25 ml Cryo-Dilution Buffer is required per sample. Use only cool buffer (4°C).

### Preparation of Working Solutions

- **1xDilution Buffer:** Dilute 10xDilution Buffer 1:10 with double distilled H<sub>2</sub>O, e.g. take 10 ml 10xDilution Buffer, add 90 ml double distilled H<sub>2</sub>O (see notes) and mix well. About 100 ml of 1xDilution Buffer is required per sample of peripheral blood. For bone marrow, cryopreserved cells and lymph nodes 50 ml of 1xDilution Buffer per sample are sufficient.

**Note:** Use 1xDilution Buffer for dilution of 10xMACS CellStain Solution.

- **1xMACS CellStain Solution:** Dilute 10xMACS CellStain Solution 1:10 with 1xDilution Buffer. About 50 ml of 1xMACS CellStain Solution is required per sample of peripheral blood. For bone marrow, cryopreserved cells and lymph nodes 25 ml of 1xMACS CellStain Solution per sample are sufficient.

- **Fast Red TR/Naphthol AS-MX Substrate Solution:** Dissolve 1 Tris/HCl tablet in 1 ml of double distilled water. Add 1 FastRed TR/Naphthol AS-MX substrate tablet to this solution and shake until tablet is dissolved completely. Prepare immediately before use.

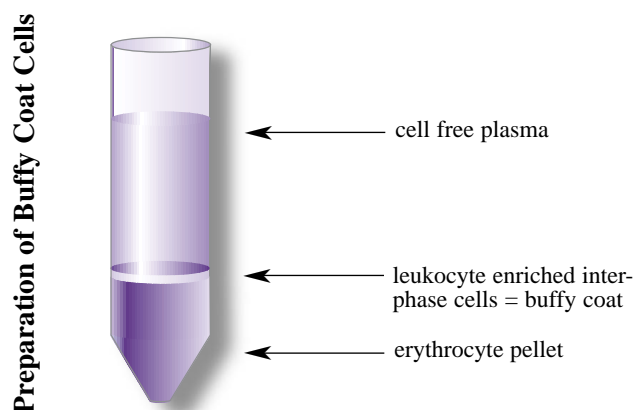
### Protocol for the Enrichment of Cytokeratin Expressing Tumor Cells

In the following protocols for preparation of peripheral blood, bone marrow, cryopreserved material, and lymph nodes are described. After fixation and washing with 1xCellStain Solution, cells can be stored in the refrigerator over night.

The volumes of buffers and reagents mentioned in these protocols are sufficient for up to 3 x 10<sup>8</sup> nucleated cells.

#### Preparation of Peripheral Blood Cells

- Centrifuge 20–40 ml anti-coagulated peripheral blood at 400xg for 35 minutes without brake (see illustration below).



- Carefully collect leukocyte enriched interphase cells (buffy coat containing about 1–3 x 10<sup>8</sup> nucleated cells) in a volume of 5 ml in a 50 ml conical tube. If the volume of the erythrocyte pellet is ≤ 5 ml, remove cell free plasma and use interphase with complete cell pellet (erythrocytes and leukocytes).
- Add 35 ml of 1xDilution Buffer to each 5 ml cell sample (final volume of 40 ml) and mix well.
- Add 5 ml of MACS CellPerm Solution, mix well and incubate cell suspension for **exactly** 5 minutes at 20–25° C.
- Add 5 ml of MACS CellFix Solution, mix well and incubate for 30 minutes at 20–25° C.
- Centrifuge cell suspension at 300xg for 10 minutes.
- Remove supernatant and resuspend cell pellet in 30 ml of 1xMACS CellStain Solution.
- (Optional) Centrifuge cell suspension at 300xg for 10 minutes.
- (Optional) Remove supernatant completely and resuspend cell pellet in 10 ml of 1xMACS CellStain Solution.
- Proceed to "Magnetic Labeling of Cytokeratin Expressing Cells".

#### Preparation of Bone Marrow Cells

- Collect 2–10 ml bone marrow containing up to 10<sup>8</sup> cells from the upper iliac crest or sternum using an aspiration needle into a 50 ml conical tube containing 2–10 ml HEPES buffered cell culture medium (i.e. IMDM) supplemented with 100 U/ml heparin. The volume of bone marrow aspirate should be about the same as the volume of medium.
- Centrifuge bone marrow cells for 10 minutes at 300xg.

- Remove supernatant completely.
- (Optional) Aspirates containing high amounts of fat should be transferred to a new tube. Therefore, resuspend cell pellet in about 1 ml HEPES buffered cell culture medium (e.g. IMDM) containing 50–100 U/ml DNase I and transfer the cells into a new tube. Rinse the old tube once with another 1 ml of the medium and add it to the cell suspension in the new tube. Adjust to a final volume of 10 ml by adding additional HEPES buffered cell culture medium containing 50–100 U/ml DNase I. Proceed to incubation for 30 minutes at 20–25° C (see below).
- Resuspend cell pellet in 10 ml of HEPES buffered cell culture medium (e.g. IMDM) containing 50–100 U/ml DNase I and incubate for 30 minutes at 20–25° C.
- Add 10 ml of 1xDilution Buffer to each 10 ml cell sample (final volume of 20 ml) and mix well.
- Add 2.5 ml of MACS CellPerm Solution, mix well and incubate cell suspension for **exactly** 5 minutes at 20–25° C.
- Add 2.5 ml of MACS CellFix Solution, mix well and incubate for 30 minutes at 20–25° C.
- Centrifuge cell suspension at 300xg for 10 minutes.
- Remove supernatant and resuspend cell pellet in 20 ml of 1xMACS CellStain Solution.
- Proceed to "Magnetic Labeling of Cytokeratin Expressing Cells".
- Add 2.5 ml of MACS CellPerm Solution to the diluted cell sample (final volume of 22.5 ml), mix well and incubate cell suspension for **exactly** 5 minutes at 20–25° C.
- Add 2.5 ml of MACS CellFix Solution, mix well and incubate for 30 minutes at 20–25° C.
- Centrifuge cell suspension at 300xg for 10 minutes.
- Remove supernatant and resuspend cell pellet in 20 ml of 1xMACS CellStain Solution.
- Proceed to "Magnetic Labeling of Cytokeratin Expressing Cells".

#### Preparation of Cryopreserved Cells

- Thaw frozen cells (usually in 1–2 ml vials containing  $10^7$ – $10^8$  cells) quickly at 40° C in a waterbath by shaking the tube. Take the tube from the waterbath when there is still a very small piece of ice in the suspension and continue shaking for some seconds until the ice has disappeared.
- Place the cells immediately at 4° C.
- Transfer the cells to a 50 ml conical tube and dilute the cells (1–2 ml) slowly with the cool (4° C) **Cryo Dilution Buffer** to a final volume of 20 ml.
- Add 2.5 ml of CellPerm Solution to the diluted cells (final volume of 22.5 ml). Mix well and incubate cell suspension for **exactly** 5 minutes at 20–25° C.
- Add 2.5 ml of MACS CellFix Solution, mix well and incubate for 30 minutes at 20–25° C.
- Centrifuge cell suspension at 300xg for 10 minutes.
- Remove supernatant and resuspend cell pellet in 20 ml of 1xMACS CellStain Solution.
- Proceed to "Magnetic Labeling of Cytokeratin Expressing Cells".
- Add 200 µl of FcR Blocking Reagent and mix well. Add 200 µl of MACS Cytokeratin MicroBeads, mix well and incubate for 45 minutes at 20–25° C.
- To detect and quantify cytokeratin expressing tumor cells by immunocytochemistry, add 100 µl of Anti-Cytokeratin-FITC and incubate for additional 10–15 minutes in the dark at 20–25° C.
- Add 4 ml of 1xMACS CellStain Solution and centrifuge cell suspension at 300xg for 10 minutes. Remove supernatant completely and resuspend the cell pellet in a final volume of 500 µl 1xMACS CellStain Solution.
- Add 10 µl of Anti-FITC Alkaline Phosphatase and incubate for 10–15 minutes in the dark at 20–25° C.
- Adjust sample to a final volume of 1 ml with 1xMACS CellStain Solution.
- Proceed to "Magnetic Separation for the Enrichment of Cytokeratin Expressing Tumor Cells".

#### Magnetic Labeling of Cytokeratin Expressing Tumor Cells in Peripheral Blood Samples

- Centrifuge cell suspension at 300xg for 10 minutes.
- Remove supernatant completely. Resuspend cell pellet in 1xMACS CellStain Solution in a final volume of 600 µl.
- Add 200 µl of FcR Blocking Reagent and mix well. Add 200 µl of MACS Cytokeratin MicroBeads, mix well and incubate for 45 minutes at 20–25° C.
- To detect and quantify cytokeratin expressing tumor cells by immunocytochemistry, add 100 µl of Anti-Cytokeratin-FITC and incubate for additional 10–15 minutes in the dark at 20–25° C.
- Add 4 ml of 1xMACS CellStain Solution and centrifuge cell suspension at 300xg for 10 minutes. Remove supernatant completely and resuspend the cell pellet in a final volume of 500 µl 1xMACS CellStain Solution.
- Add 10 µl of Anti-FITC Alkaline Phosphatase and incubate for 10–15 minutes in the dark at 20–25° C.
- Adjust sample to a final volume of 1 ml with 1xMACS CellStain Solution.
- Proceed to "Magnetic Separation for the Enrichment of Cytokeratin Expressing Tumor Cells".

#### Magnetic Labeling of Cytokeratin Expressing Tumor Cells in Samples of Bone Marrow, Cryopreserved Cells and Lymph Nodes

#### Preparation of Cells from Lymphoid Tissue

- Isolate single cell suspension containing up to  $10^8$  nucleated cells from lymphoid tissue by standard preparation method.<sup>3</sup>
- Centrifuge cells for 10 minutes at 300xg in a 50 ml conical tube.
- Remove supernatant completely, resuspend cell pellet in 1xDilution Buffer in a final volume of 20 ml and mix well.
- Centrifuge cell suspension at 300xg for 10 minutes.
- Remove supernatant completely. Resuspend cell pellet in 1xMACS CellStain Solution in a final volume of 300 µl.
- Add 100 µl of FcR Blocking Reagent and mix well. Add 100 µl of MACS Cytokeratin MicroBeads, mix well and incubate for 45 minutes at 20–25° C.
- To detect and quantify cytokeratin expressing tumor cells by immunocytochemistry, add 50 µl of Anti-Cytokeratin-FITC and incubate for additional 10–15 minutes in the dark at 20–25° C.
- Add 3 ml of 1xMACS CellStain Solution and centrifuge cell suspension at 300xg for 10 minutes. Remove supernatant completely and resuspend the cell pellet in a final volume of 250 µl 1xMACS CellStain Solution.
- Add 5 µl of Anti-FITC Alkaline Phosphatase and incubate for 10–15 minutes in the dark at 20–25° C.
- Adjust sample to a final volume of 500 µl with 1xMACS CellStain Solution.

- Proceed to "Magnetic Separation for the Enrichment of Cytokeratin Expressing Tumor Cells".

### Magnetic Separation for the Enrichment of Cytokeratin Expressing Tumor Cells

- Place a positive selection column type MS<sup>+</sup>/RS<sup>+</sup> (with the RS<sup>+</sup> column adapter) in the magnetic field of the MACS separator. Prepare the column by washing with 500 µl of degassed 1xDilution Buffer.
- Pass cells through 30 µm nylon mesh or Pre Column Separation Filter to remove any clumps. Wet filters with degassed 1xDilution Buffer before use.
- Apply cells onto the column, allow cells to pass through the column and wash with 3 x 500 µl of degassed 1xDilution Buffer.
- Remove column from separator, place column on a suitable tube, pipette 1 ml of degassed 1xDilution Buffer on top of column and elute retained cells using the plunger supplied with the column.
- To achieve a higher enrichment rate, apply the eluted cells onto a new freshly prepared positive selection column, wash the column with 1 x 500 µl of degassed 1x Dilution Buffer and 1 x 500 µl of degassed PBS. Elute retained cells in 500 µl of degassed PBS.
- Proceed to "Detection and Quantification of Cytokeratin Expressing Tumor Cells by Immunocytochemistry".

### Detection and Quantification of Cytokeratin Expressing Tumor Cells by Immunocytochemistry

- Spin the magnetically enriched cell fraction on a slide using a centrifuge (e.g. HETTICH Cyto-System). Air dry slide for 2–24 hours at 20–25° C.
- Using a pen (e.g. DAKO PEN, diamond pen) apply a line around the cell area on the slide.
- Wash slide once for one minute in PBS.
- Add 30–50 µl of freshly prepared Fast Red TR/Naphthol AS-MX Substrate Solution to cell spots on the slide. Incubate slide for 15 minutes in humidity chamber at 20– 25° C.
- Wash slide for one minute in double distilled water and thereafter air dry.
- Analyze slide for cytokeratin positive cells microscopically.
- For additional cytomorphological investigation slide can be stained with hemalum solution. Therefore dilute Meyer's hemalum solution 1:2 in 100 mM of TrisHCl, pH 8.2 and filter the diluted hemalum solution.
- Wash slide for 1 minute in PBS in staining trough.
- Incubate slide for 30 seconds in diluted hemalum solution in staining trough.
- Wash slide twice for 1 minute in double distilled water in staining trough and air dry thereafter.
- Mount slide with Kaisers glycerol gelatin.

### Important Notes

- ▲ Prepare samples for magnetic separation within 24 hours.
- ▲ Keep cells at temperatures below 25° C before processing.

- ▲ When the volume of the blood cell pellet is ≤ 5 ml use the whole cell pellet.
- ▲ When using carcinoma cell line cells for spiking experiments the adherent layer in the culture vessel should not be completely confluent. Otherwise it might be difficult to get a single cell suspension.
- ▲ Blood and bone marrow samples containing fat have to be washed fat free before starting the procedure.
- ▲ We strongly recommend processing one positive (e.g. peripheral blood cells spiked with carcinoma cell line cells) and one negative control (e.g. peripheral blood cells from healthy individuals) in parallel to each series of samples.
- ▲ 10x Dilution Buffer and 10xMACS CellStain Solutions should be freshly diluted prior to use.
- ▲ For running the columns use degassed buffer only! Degassing of buffer can be performed using a vacuum. Excess of gas in buffer will form bubbles in the matrix of the column during separation. This may lead to clogging of the column and decreases the quality of separation.
- ▲ In order to reduce background during detection and quantification of enriched cytokeratin expressing tumor cells, we recommend passing the double distilled water you use for preparation of 1xDilution Buffer through a 0.22 µm filter.
- ▲ After fixation and washing with CellStain Solution, cells can be stored in the refrigerator overnight.
- ▲ Increased temperature and prolonged incubation time for magnetic labeling may lead to unspecific cell labeling.

### References

1. Heatley, M; Maxwell, P; Whiteside, C; Toner, P (1995) Cytokeratin intermediate filament expression in benign and malignant breast disease. J. Clin Pathol. 48: 26-32.
2. Makin, C, Bobrow, L, Budmer, W (1984) Monoclonal antibody to cytokeratin for use in routine histopathology. J. Clin. Pathol. 37: 975.
3. Coligan, JE; Kruisbeek, AM; Margulies, DH; Shevach, EM; Strober, W; (Eds.): Current Protocols in Immunology, John Wiley & Sons, Inc. (1994).

### Warning

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

Do not touch SIGMA FAST™ Fast Red TR/Naphthol AS-MX substrate tablets with your fingers and avoid any skin contact. SIGMA FAST™ Fast Red TR/Naphthol AS-MX substrate tablets and the solutions prepared therefrom are toxic.

MACS CellFix Solution contains formaldehyde and should be disposed of properly.

### Warranty

The products sold hereunder are warranted only to be free from defects in workmanship and material at the time of delivery to the customer. MILTENYI BIOTEC GmbH makes no warranty or representation, either expressed or implied, with respect to the fitness of a product for a particular purpose. There are no warranties, expressed or implied, which extend beyond the Technical Specifications of the products. MILTENYI BIOTEC GmbH's liability is limited to either replacement of the products or refund of the purchase price. MILTENYI BIOTEC GmbH is not liable for any property damage, personal injury or economic loss caused by the product.