ProMab Biotechnologies, Inc.

Protocol

Magnetic Cell Selection and Separation of Human CD326+ Cells

OVERVIEW

The MAG-iso™ Human CD326+ Cancer Cells Isolation Kit (cat. # K10109, ProMab Biotechnologies) is designed to isolate CD326+ (EpCAM) human MCF7 breast cancer cells from a mixture of 1/3 MCF7 cells and 2/3 of PLC/PRF5 liver cancer cells using positive selection. The resulting cell preparation is highly enriched for CD326+ cells. Purity of recovered CD326+ cells can be up to 80% and will vary depending on the preparation.

MATERIALS REQUIRED

1. Magnetic Separator like MagCellect Magnet (R&D System, Catalog # MAG997)
2. 12 x 75 mm (5 mL) tubes (Falcon, Catalog # 352008 or equivalent)
3. 50mL polypropylene centrifuge tubes (Santa Cruz Biotechnology, Inc, Catalog # sc-200251, or equivalent)
4. Sterile serological and Pasteur pipettes or transfer pipettes
5. Bench top centrifuge
6. 2-8° C refrigerator
7. Deionized or distilled water

Cell Selection Principle

1. Positive selection of CD326+ cells is achieved by incubation with biotinylated anti-Human CD326 monoclonal antibody.
2. CD326 monoclonal antibody bound cells are then magnetically tagged with MAG-iso™-Streptavidin 200nm.
3. Magnetically tagged CD326+cells are then retained in the magnetic column. (These are the desired cells); unwanted/untagged cells run through.
4. Magnetically tagged cells are then isolated using magnetic separation. CD326+ tagged cells will migrate toward the magnet (these are the desired cells); unwanted/untagged cells remain in suspension

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Cell Selection Capacity

Separator	Max No. of labeled cells	Max No. of total cells
R&D Magnet	*1.7x10^6	*2x10^6 Cell

*: The Max No. of cells will vary by ±20% depending on the preparation.

Components of Kit (up to 100 tests, 109 cells).

1. Biotinylated anti-Human CD326 Antibody (Biolegend Catalog#: 324215) – 0.2mL (for up to 400 tests with 1x10^6 cells per test).
2. MAG-iso^{TM} Streptavidin 200nm - 1mL proprietary formulation
3. 1X Selection Buffer - 50 mL proprietary formulation.
4. DRNase (proprietary formulation of DNase I and RNase) – 400µL (Part M10001)

Storage

Reagents are stable for 12 months from the date of receipt when stored in the dark at 2 - 8° C. DO NOT FREEZE.
DRNase can be stored in -20° C.

Reagent Preparation

1x Selection Buffer: Prepare 1 L of 1x Selection Buffer by dissolving and mixing 5 g of BSA (Amresco Catalog# 0332) and 4 ml of 0.5 M EDTA (Promega Catalog# V4231) in 1 L of 1x PBS (prepared from 10x PBS; Teknova Catalog# P0195). The 1x Selection Buffer is stable for 6 months at 4°C and should be kept on ice or at 4°C but can be kept at room temperature in Magnetic separation step.

Cell Selection Procedure

I. **Cell Preparation**: Cells and reagents should be kept cold using an ice bath or a refrigerator unless otherwise specified. Incubations must be carried out at 2 - 8°C in a refrigerator and not in an ice bath to avoid excessively low temperatures that can slow the kinetics of the optimized reactions.

Prepare the reaction buffer ahead of time and keep it refrigerated or on ice - see Reagent Preparation.

1. To a 50 mL conical tube add 30µL formulated DRNase
2. Prepare cell suspension from fresh MCF7 and PLC/PRF5 adherent cell
3. Transfer cell suspension containing 1/3 of MCF7 cells and 2/3 of PLC/PRF5 cells to the 50ml conical tube.
4. Slowly add 15mL pre-warmed (37°C) DMEM or RPMI medium (with 10% FBS) drop wise to the cells.

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5. Centrifuge cell suspension at 4°C for 15 minutes at 200g.
6. Carefully remove all but approximately 100µL of the supernatant.
7. Gently resuspend cell pellet in 0.5 mL of 1x Selection Buffer (pre-chilled to 4°C) and transfer to a 1.5 ml micro-centrifuge tube.
8. Pass the cell suspension through a 30-50µm nylon cell strainer.

Cells must be resuspended in cold reaction buffer prior to the antibody selection procedure. Buffer has to be kept on ice at all times.

NOTE: For downstream applications that are sensitive to DRNase (eg. hematopoietic colony assays), wash cells once in the appropriate assay buffer (without DRNase) before continuing.

II. Positive selection of CD326+ cells

1. Transfer desired amount CD326+ cells to a micro-centrifuge tube.
2. *Fc domain blocking (Optional)*: In some applications, this step is required to minimize non-specific binding of the antibodies via their Fc domain to the Fc receptors (FcR) present on various cell types.
3. Add 1-10 µg of IgG or Fc blocking specific antibody (in a volume not exceeding 100 µL) per $1x10^7$ cells and incubate 5-10 minutes at 2 - 8° C.
4. Add 2 µL (~2 ug) of biotinylated anti-human CD326+ antibody per $1x10^7$ cells. (Biolegend Catalog#: 324215) to the tube.
5. Gently mix the cell-antibody suspension, avoiding formation of bubbles, and incubate at 2-8°C on a rotator for 15 minutes.
6. After incubation, dilute the cell suspension with 6 mL of cold 1x Selection Buffer. Aliquot into 4 Eppendorf tubes and centrifuge at 4°C for 6 minutes at 300g. Repeat washing step once more.
7. Completely remove the supernatant and gently resuspend the cell pellet with 0.5 mL of cold 1x Selection Buffer.
8. Add 50 µL of 1:6 dilution (8.5 µl in 50 µl) of MAG-iso™ Streptavidin 200nm (Catalog# 03121) to the cell suspension. Mix gently and incubate at 2 - 8° C on a rotator in a refrigerator for 15 minutes.
9. After incubation, dilute the cell suspension with 6 mL of cold 1x Selection Buffer. Aliquot into 4 Eppendorf tubes and centrifuge at 4°C for 6 minutes at 300g. Repeat washing step once more.
10. Completely remove supernatant and gently resuspend cell pellet with 0.5 mL of cold 1x Selection Buffer and transfer contents into a 5mL Falcon tube. Add another 2 ml of cold 1x Selection Buffer to the tube.

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III. Magnetic Separation with R&D Magnet

1. Place the 5 mL Falcon tube in the magnetic fields of R&D Magnet or a suitable Separator.
2. Incubate for 6 minutes at room temperature (18-25 °C).
3. Magnetically tagged cells will migrate toward the magnet (these are desired CD326+ cells), leaving the unwanted/non-specific cells in suspension
4. While the tube remains in the magnet, carefully remove all the reaction supernatant and save as Flow Through.
5. Remove the tube containing the desired magnetically bound CD326+ cells from the magnet, and gently resuspend pellet in 2 mL of cold 1x Selection buffer.
6. Repeat steps 1. through 4. 2-3x with the positively selected (bound) cell fraction following cell re---suspension; save rest of the reaction supernatant as Wash.
7. Upon final wash, resuspend cell pellet in 1mL of 1x Selection Buffer.
8. The resulting bound cells contain the desired and enriched CD326+ cells, where Flow through and Wash contain the undesired CD326- cells.
9. Centrifuge the fractions at 300 x g for 6 minutes (save the pellet for FACS analysis).
10. Cells are now ready for further experimentation or FACS analysis.