ProMab Biotechnologies, Inc.

Protocol

Magnetic Cell Selection and Separation of Human CD133+ Cells

OVERVIEW

The MAG-iso™ Human CD133+ Cancer Cells Isolation Kit (cat. # K10108, ProMab Biotechnologies) is designed to isolate CD133 + PLC/PRF/5 liver cancer cells using positive selection. The resulting cell preparation is highly enriched for CD133+ cells. Purity of recovered CD133+ cells can be up to 95% and will vary depending on the preparation.

MATERIALS REQUIRED

1. Biotinylated anti-Human CD133 Antibody (Miltenyi Catalog#: 130-090-664) – 1mL (for up to 20 tests with 5x106 cells per test).
2. PM Magnet (ProMab Biotechonologies, Inc., (Catalog # M10000) or equivalent (BD Catalog # 557983)
3. 12 x 75 mm (5 mL) tubes (Falcon, Catalog # 352008 or equivalent)
4. 17 x 100 mm (15 mL) polystyrene round bottom tubes (Falcon, Catalog # 352006, or equivalent)
5. Sterile serological and Pasteur pipettes or transfer pipettes
6. Bench top centrifuge
7. 2 - 8° C refrigerator
8. Deionized or distilled water
9. MAG-iso™-Streptavidin (Part B10001) - 1mL proprietary formulation (sufficient for 20 selections).
10. DRNase (proprietary formulation of DNase I and RNase) –600µL (Part DR10101) (30µL per test)

Cell Selection Principle

1. Positive selection of CD133+ cells is achieved by incubation with biotinylated anti-Human CD133 monoclonal antibody.
2. CD133 monoclonal antibody bound cells are then magnetically tagged with MAG-iso™-Streptavidin.
3. Magnetically tagged cells are then isolated using magnetic separation. CD133+ 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	Ma x No. of labeled cells	Ma x No. of total cells
PM Magnet	*3.5x10^6	*5x10^6 Cell

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

Components of Kit (up to 20 tests, 5 x10^6 cells per test).

1. MAG-isoTM-Streptavidin (Part B10002) - 1mL proprietary formulation (sufficient for 20 selections).
2. 10X MAG-iso^{TM} Buffer (Part S10001) - 50 mL proprietary formulation.
3. DRNase (proprietary formulation of DNase I and RNase) – 4 00µL (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 final solution will be Phosphate buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA) and 2 mM EDTA. Degas before use. 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.

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A. Preparing a single cell suspension from frozen cells

1. To a 50 mL conical tube, add 30µL formulated DRNase.
2. Transfer the cell suspension to the 50ml conical tube.
3. Slowly add 10mL pre-warmed (37°C) DMEM medium (with 10% FBS) drop wise to the cells.
4. Centrifuge cell suspension at 200 x g at 4°C for 15 minutes.
5. Carefully remove all but approximately 100µL of the supernatant using a pipette.
6. Gently resuspend the cell pellet in 10mL of fresh medium (pre-chilled to 4°C) to the tube.
7. Centrifuge the suspension at 200 x g at 4°C for 15 minutes.
8. Remove media and resuspend final pellet up to 5 x10^6 in 500µL of Selection Buffer.
9. Pass the suspended cells through a 30-50µm nylon cell strainer.

Cells must be resuspended in cold reaction buffer prior to the antibody selection procedure. Buffer must 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.

B. Preparing a single cell suspension from fresh adherent cells

NOTE: Cells reaching 80-100% confluence are ready for harvest and can be used for selection.

1. Remove media and rinse adherent cells with PBS.
2. Treat cells with 3mL 1x Trypsin EDTA (0.05%Trypsin/0.53mM EDTA in HBSS) for 2-3 min.
3. Break cell clumps by serological pipetting 8-10 times.
4. Add 12mL culture medium; break the cell clumps to single cell suspension by serological pipetting 8-10 times.
5. Centrifuge in 50ml conical tube for 5min at 300 x g.
6. Remove media and resuspend final pellet up to 5 x10^6 in 500µL of Selection Buffer.
7. Pass the suspended cells through a 30-50µm nylon cell strainer.

C. Preparing a single cell suspension from fresh suspension culture

1. Harvest suspension culture into 50ml conical tube and centrifuge for 5 min at 300 x g.
2. Remove media and rinse pellet with PBS.
3. Centrifuge for 5 min at 300 x g; remove the PBS.
4. Treat the cells with 1mL 1x Trypsin EDTA (0.05%Trypsin/0.53mM EDTA in HBSS) for 2-3 min.
5. Break cell clumps by pipetting 8-10 times with P1000 pipette, or until a single cell suspension is achieved.
6. Add 10mL culture medium; centrifuge for 15 min at 200 x g at 4°C.
7. Remove media and resuspend final pellet up to 4 x10^6 in 500µL of Selection Buffer.
8. Pass the suspended cells through a 30-50µm nylon cell strainer.

NOTE: 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

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II. Positive selection of CD133+ cells

1. Transfer desired amount CD133+ cells to a micro-centrifuge tube.

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. 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.

2. Add 50µL of biotinylated anti-human CD133+ antibody for $5x10^6$ cells. (Miltenyi Catalog#: 130-090-664) to the tube.
3. Gently mix the cell-antibody suspension, avoiding formation of bubbles, and incubate at 2-8°C on a rotator for 15 minutes.
4. 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.
5. Completely remove the supernatant and gently resuspend the cell pellet with 0.5 mL of cold 1x Selection Buffer.
6. Add 50 µL of 1:6 dilution (8.5 µl in 50 µl) of MAG-iso™ Streptavidin 200nm (Catalog# 03121) to the cell suspension 5x106 cells. Mix gently and incubate at 2 -8° C on a rotator in a refrigerator for 15 minutes.
7. After incubation, dilute cell suspension to 10mL with cold Selection Buffer, and centrifuge at 4°C for 15 minutes at 300 x g.
8. Completely remove supernatant and gently resuspend cell pellet with 3mL of cold MAG-iso™ Buffer and transfer contents into a 5mL Falcon tube.

III. Magnetic Separation with Magnet

1. Place the 5 mL Falcon tube in the magnetic fields of PM Magnet or a suitable Separator.
2. Incubate for 6 minutes at room temperature (18-25 °C). Magnetically tagged cells will migrate toward the magnet (these are desired CD133+ cells), leaving the unwanted/non-specific cells in suspension.
3. While the tube remains in the magnet, carefully remove all the reaction supernatant and save as Flow Through.
4. Remove the tube containing the desired magnetically bound CD133+ cells from the magnet, and gently resuspend pellet in 2 mL of cold 1x Selection buffer.
5. 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.
6. Upon final wash, resuspend cell pellet in 1mL of 1x Selection Buffer. The resulting bound cells contain the desired and enriched CD133+ cells, where Wash contain the undesired CD133- cells.
7. Centrifuge the fractions at 300 x g for 6 minutes (save the pellet for FACS analysis).
8. Cells are now ready for further experimentation or FACS analysis