



Magnetic Cell Selection and Separation of Human CD24^{-/low}CD44⁺ Cells

OVERVIEW

The MAG- iso™ Human CD24^{-/low}CD44⁺ Cancer Cells Isolation Kit (cat. # K10103, ProMab Biotechnologies) is designed to isolate CD24^{-/low} CD44⁺ human cancer stem cells using positive selection. The resulting cell preparation is highly enriched for CD24^{-/low}CD44⁺ cells. Purity of recovered CD24^{-/low} CD44⁺ cells can be up to 90%-99% and will vary depending on the preparation.



MATERIALS REQUIRED

1. Magnetic Separator like MagCollect Magnet (R&D Systems, Catalog # MAG997)
2. 12 x 75 mm (5 mL) tubes (Falcon, Catalog # 352008 or equivalent)
3. 50mL polypropylene centrifuge tubes (Santa Cruz Biotechnology, Inc, Cat. # 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. Negative selection of CD24⁺ cells is first achieved by incubation with biotinylated anti-Human CD24 monoclonal antibody.
2. CD24 antibody bound cells are then magnetically tagged with MAG-iso™ -Streptavidin.
3. Magnetically tagged cells are then isolated away using magnetic separation. CD44⁺ tagged cells will migrate toward the magnet (unwanted CD24⁺ cells); desired CD24^{-/low} cells remain in suspension.

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4. Positive selection of CD44⁺ cells from the remaining population of suspension cells then proceeds by incubation with biotinylated anti-Human CD44 monoclonal antibody.
5. CD44 monoclonal antibody bound cells are then magnetically tagged with MAG-isoTM-Streptavidin.
6. Magnetically tagged cells are then isolated using magnetic separation. CD24^{-/low}CD44⁺ tagged cells will migrate toward the magnet (these are the desired cells); unwanted/untagged cells remain in suspension

Cell Selection Capacity

Separator	Max No. of labeled cells	Max No. of total cells
PM Magnet	*2x10 ⁶	*5x10 ⁶

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

Components of Kit (up to 20 tests, 10⁹ cells).

1. Biotinylated anti-Human CD24 Antibody (Part C10106) - 1mL (for up to 20 tests with 5x10⁶ cells per test).
2. Biotinylated anti-Human CD44 Antibody (Part C10108) - 1mL (for up to 20 tests with 5x10⁶ cells per test).
3. MAG-isoTM-Streptavidin (Part B10002) - 2mL proprietary formulation (sufficient for 20 double selections).
4. 10X MAG-isoTM Buffer (Part S10001) - 50 mL proprietary formulation.
5. DRNase (proprietary formulation of DNase I and RNase)-700 μ L (Part M10001).

Storage

Reagents, except DRNase, 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 for long-term storage.

Reagent Preparation

1x MAG- isoTM Buffer:

Prepare 20 mL of 1x MAG- isoTM Buffer for each sample by mixing 2 mL of 10X MAG- isoTM Buffer with 18 mL of sterile deionized or distilled water. The 1x MAG- isoTM 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



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Cell Selection Procedure (5x10⁶ cells/mL*)

*Procedure has been used to select cells from a starting population as low as 1x10⁶ cells/mL

- 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.

A. Preparing a single cell suspension from frozen cells

NOTE: When thawing units with large cell numbers (>10x10⁶), treat the cells with DRNase.

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 x10⁶ 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 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.

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.





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5. Centrifuge in 50ml conical tube for 5min at 300 x g.
6. Remove media and resuspend final pellet up to 5×10^6 to a concentration of 1×10^7 cells/mL in 500 μ L of in MAG-iso™ Buffer Selection Buffer.
7. Pass the suspended cells through a 30-50 μ m 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 the pellet in MAG- iso™ Buffer to a concentration of 8×10^6 cells/mL.
8. Pass the suspended cells through a 30-50 μ m nylon cell strainer.

NOTE: Regardless of which preparation is undertaken, it is imperative to ensure a single-cell suspension by passing cells through a 30-50 μ m nylon cell strainer.

NOTE: Cells must be suspended in cold reaction buffer to a concentration of 1×10^7 cells/mL prior to beginning the antibody selection procedure.

(Optional): Fc domain blocking

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.

1. Transfer 0.5mL suspended cells (5×10^6 adherent or, 4×10^6 suspension cells) into an Eppendorf tube.
2. Add 1-10 μ g of IgG or Fc blocking specific antibody (in a volume not exceeding 100 μ L) per 1×10^7 cells and incubate 5-10 minutes at 2 - 8° C.

II. Negative selection of CD24^{-/low} cells

1. Transfer desired number of total cells to a micro-centrifuge tube to end with the necessary amount of CD24^{-/low} CD44⁺ cells.
2. Add to the Eppendorf tube 50 μ L of biotinylated anti- human CD24 antibody (Part C10106) per 5×10^6 cells.
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 1mL of cold 1x MAG-iso™ Selection Buffer. Aliquot into Eppendorf tubes and centrifuge at 4°C for 5 minutes at 300 x g or in a single-speed clinical bench top centrifuge for 1 minute.



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5. Remove the supernatant. Resuspend the cell pellet by gently pipetting 1.5 mL of cold 1x MAG-iso™ Buffer, and centrifuge in a refrigerated Eppendorf centrifuge at 300 x g for 5 minutes or in a single-speed clinical bench top centrifuge for 1 minute.
6. **Completely** remove the supernatant. Gently resuspend the cell pellet with 0.5 mL of cold 1x Selection Buffer.
7. Add 50 µL MAG- iso™ -Streptavidin (Part B10002) to the cell suspension. Mix gently and incubate at 2 - 8° C on a rotator in a refrigerator for 15 minutes.
8. At the end of the incubation period, dilute the cell content to 1.5mL with cold 1x MAG- iso™ Buffer, centrifuge in a refrigerated Eppendorf centrifuge at 300 x g for 5 minutes or in a single-speed clinical bench top centrifuge for 1 minute.
9. **Completely** remove the supernatant. Resuspend the cell pellet by gently pipetting 1.5mL of cold 1x MAG- iso™ Buffer, and centrifuge in a refrigerated Eppendorf centrifuge at 300 x g for 5 minutes or in a single-speed clinical bench top centrifuge for 1 minute.
10. **Completely** remove the supernatant and resuspend the cell pellet by gently pipetting 3mL of cold 1x MAG- iso™ Buffer and transfer the solution into a 5mL Falcon tube.

III. Magnetic Separation

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 the unwanted CD24⁺ cells), leaving the desired CD24⁻/low cells in suspension.
4. While the tube remains in the magnet, carefully remove all the reaction supernatant and save to a 15mL tube as Flow Through - move to steps 6 to continue with processing of the reaction supernatant.
5. Remove the tube containing undesired CD24⁺ magnetically bound cells from the magnet and resuspend the cells in 1mL of reaction buffer by gently pipetting up and down (save the fraction for a separation quality test using FACS analysis).
6. Repeat steps 2. through 4. 2-3 times with the bound cell fraction following resuspension if desired for higher rate of recovery, but not recommended for regular selection. Save the fractions for a separation efficiency test using FACS analysis.
7. Centrifuge the 15mL tube from step 4. in a refrigerated Eppendorf centrifuge at 300 x g for 5 minutes; resuspend the CD24⁻/low cell pellet by gently pipetting 0.5 mL of cold 1x MAG-iso™ Buffer.
8. Cells are now ready for additional selection, further experimentation or analysis.



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IV. Positive selection of CD44 cells

1. Add 50 μ L of biotinylated anti-human CD44 antibody (Part C10108) to the tube from Section III step 8.
2. Gently mix the cell-antibody suspension, avoiding formation of bubbles, and incubate at 2 - 8° C on a rotator for 15 minutes.
3. At the end of the incubation period, dilute the cell suspension by adding 1mL of cold 1x MAG-iso™ Buffer, and centrifuge in a refrigerated Eppendorf centrifuge at 300 x g for 5 minutes or in a single-speed clinical bench top centrifuge for 1 minute.
4. Remove the supernatant and resuspend the cell pellet by gently pipetting 1.5 mL of cold 1x MAG-iso™ Buffer into the tube, and centrifuge in a refrigerated Eppendorf centrifuge at 300 x g for 5 minutes or in a single-speed clinical bench top centrifuge for 1 minute.
5. Completely remove the supernatant and resuspend the cell pellet by gently pipetting 0.5 mL of cold 1x MAG-iso™ Buffer.
6. Add 50 μ L MAG-iso™-Streptavidin (Part B10002) to the cell suspension. Mix gently and incubate at 2 - 8° C on a rotator in a refrigerator for 15 minutes.
7. At the end of the incubation period, dilute the cell content to 1.5mL with cold 1x MAG-iso™ Buffer, centrifuge in a refrigerated Eppendorf centrifuge at 300 x g for 5 minutes or in a single-speed clinical bench top centrifuge for 1 minute.
8. Remove the supernatant and resuspend the cell pellet by gently pipetting 1.5mL of cold 1x MAG-iso™ Buffer, and centrifuge in a refrigerated Eppendorf centrifuge at 300 x g for 5 minutes or in a single-speed clinical bench top centrifuge for 1 minute.
9. Completely remove the supernatant and resuspend the cell pellet by gently pipetting 3mL of cold 1x MAG-iso™ Buffer and transfer the cell into a 5mL Falcon tube.

V. Magnetic Separation

1. Place the 5 mL Falcon tube from Section IV step 9 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 CD24^{-/low} CD44⁺ 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 to a 15ml tube as Flow Through.
5. Remove the tube containing the desired magnetically bound CD24^{-/low} CD44⁺ cells from the magnet, and gently resuspend pellet in 3 mL of cold 1x Selection buffer by gently pipetting up and down.
6. Repeat steps 2. through 4. 2-3x with the positively selected (bound) cell fraction following cell resuspension; save all the reaction supernatant to the 15ml tube.
7. The resulting bound cells in the pellet contains the desired and enriched CD24^{-/low} CD44⁺ cells. The resultant supernatant fraction obtained from steps 4. to 6. contains the undesired CD44⁻ cells.



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8. Centrifuge the fractions at 300 x g for 5 minutes.
9. The resulting bound cells contain the desired and enriched CD24^{-/low}CD44⁺ cells, save the pellet for FACS analysis). The Flow Through and the final Wash contain the undesired CD44⁻ cells and can be analyzed or discarded following lab protocol.
10. Cells are now ready for further experimentation or FACS analysis.

VI. Cell Harvesting

1. Depending upon the intended use of the cells, resuspend the beads fraction and/or the pellet from the supernatant fraction with your application buffer.
2. For FACS analysis, resuspend the beads fraction and the pellet from the supernatant fraction with 1.5ml cell staining buffer (Part S10002; not included); centrifuge at 300 x g for 3 min; remove the supernatant; (continue to Cell Staining Procedure).
3. For cryopreservation, re-suspend the beads and the pellet in 1x DMEM or RPMI medium (continue to Cell Cryopreservation Procedure).

