2

Immunomagnetic Cell Separation

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1. Introduction

In metastasis research, it may sometimes be necessary to separate populations of tumor cells from a mixed cell population such as a tumor, peripheral blood, or bone marrow. In addition, the normal counterparts of populations of tumor cells can be separated to allow direct comparisons to be made ***(1)***. In recent years magnetic bead separation techniques have become increasingly popular for these purposes.

Immunomagnetic separation methods are based on the attachment of small magnetizable particles to cells via antibodies or lectins. When the mixed population of cells is placed in a magnetic field, those cells that have beads attached will be attracted to the magnet and may thus be separated from the unlabeled cells.

Several makes of bead are available, some of which are designed specifically for cell sorting, and others that are designed for purifying molecules (particularly nucleic acids) but that may be adapted for cell sorting if necessary. The different types of beads work on the same principle, but the strength of the magnetic field required to separate the cells differs depending on the size of the beads. Of the larger beads (>2 μm), the most commonly used type is the range produced by Dynal (Dynal [UK] Ltd., Wirral, Mersyside, UK; Dynal, Inc., Lake Success, NY). The smaller beads (<100 nm) represented by the MACS system produced by Miltenyi Biotech (Miltenyi Biotech Ltd., Bisley, Surrey, UK; Miltenyi Biotech Inc., Auburn, CA) require a more complicated separation apparatus. Details of each type of bead together with advantages and disadvantages of each system are described below.

Dynabeads are 4.5-μm superparamagnetic beads; that is, they have no residual magnetism outside a magnetic field. An iron-containing core is surrounded by a thin polymer shell to which biomolecules may be adsorbed.

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The beads can be coated in primary antibodies, species-specific antibodies, lectins, enzymes, or streptavidin. The beads may be attached to cells via a coating of primary antibodies specific for the cell type using beads bought ready coated, or using beads coated by the user with their own antibody. Alternatively the cells, rather than the beads, may be labeled with a primary antibody, and then species-specific secondary antibody-coated beads added. Similarly, streptavidin-coated beads can be used in conjunction with biotinylated primary or secondary antibodies. The cells, surrounded by a "rosette" of beads, may then be separated from the unlabeled population in a magnetic field using a relatively small (but powerful) magnet produced by Dynal.

If no antibody is available that specifically identifies a cell type in a heterogeneous population, the cells may still be separated using the "negative sorting" method. In this case, all the unwanted cell types are immunomagnetically labeled, a process that may require a cocktail of antibodies. The labeling procedure is the same as for positive sorting except that the unlabeled fraction of the cell population is retained and the labeled cells are discarded.

The range of precoated beads available includes those coated in antibodies specific for human B (CD19) and T cells (CD2 and CD3) and T-cell subsets (CD4 and CD8), hematopoietic progenitor cells (CD34), and monocytes (CD14). For metastasis research two types of beads are available to separate tumor cells from blood or bone marrow. For epithelial tumors, beads coated with antibodies against the human epithelial antigen are available. Nonepithelial tumors, however, require negative selection using anti-CD45-coated beads to remove all the leukocytes. It is possible to separate cells not only from blood, but also from a primary tumor and/or arising metastases by first disaggregating the tumor to form a single-cell suspension and then labeling the cells with a suitable antibody. Frequently in tumor samples only a small number of cells are present, and the process of cell sorting, requiring several washing steps, may result in unacceptable cell losses. In this case, it is worth precoating the beads with the antibody rather than labeling the cells and then using species-specific secondary antibody-coated beads, to limit the number of washing steps required, and thus reduce possible cell loss.

The beads may be left attached to the cells even if the cells are to be subsequently cultured. If the density of the beads is too great, however, they may interfere with cell attachment and growth, and should be removed. It is also desirable to remove the beads from the cell surface if the cells are subsequently to be used in experiments to investigate cell–cell interactions. Several options are available for removing the beads. First, some precoated beads (anti-human CD4, CD8, CD19, CD34, and antimouse CD4) may be removed using a polyclonal anti-Fab antibody, DETACHaBEAD, which competes with antibody–antigen binding to release the antibody and bead from the cell. Second, a

new type of bead has been produced that may be used for any cell separation, and that is specifically designed to be released from the cells. CELLection beads are available both primary antibody coated and for use with any mouse primary antibody or biotinylated antibody. Antibodies are attached to the surface of these beads via a DNA linker, which may be cleaved after the cells have been isolated by the addition of a DNase releasing buffer. Thus, although the beads are removed, the cells retain attached antibodies.

The MACS separation system ***(2)*** uses particles consisting of iron oxide and polysaccharide. These beads are approx 50 nm in diameter, and they require a far stronger magnetic field than that provided by the Dynal magnet to separate cells. As with Dynabeads, cells may be negatively or positively sorted from a population using the MACS separation system. A large range of primary antibody-coated beads is available to sort leukocyte subsets, fibroblasts, endothelial cells, epithelial cells, and apoptotic cells. Alternatively, the cells may be labeled with primary antibodies followed by species-specific antibody-coated MACS beads. The labeled cell suspension is then placed in a separation column in a strong magnetic field. The column contains a plastic-covered ferromagnetic core through which the cell suspension can flow. The flow rate is governed by the size of the hole at the base of the column or by an attached needle (depending on the column type). The labeled cells are retained within the column as long as it remains in the magnetic field, and unlabeled cells flow through and can be collected. The column may then be removed from the magnetic field, allowing the positive cells to be eluted. Following cell separation, MACS beads are internalized by the cells, and so they do not need to be removed because they do not interfere with cell attachment to the culture surface or with cell–cell interactions. It may be necessary to remove the beads, however, if a subset of cells are to be resorted from a population already sorted using MACS beads. A bead removal reagent is available for this purpose that enzymatically removes the MACS beads and allows the cells to be relabeled with another marker and sorted again.

The two bead separation systems have advantages and disadvantages. Until recently, it was preferable to separate cells using the MACS system if they were to be subsequently used in studies of cell–cell interactions. The development of removable types of Dynabeads means that this is no longer the case. Dynabeads are not suitable for every type of cell separation, however, because, in rare cases, they have been shown to strip the antigen off the surface of cells, making cell separation impossible ***(3)***. The main disadvantage of the MACS system is that initial costs are higher to purchase the separation magnet, and running costs include not only the price of the beads, but also replacement columns. In comparison to the running costs of a fluorescence-activated cell sorter (FACS) (methodology described in Chapter 1 by Davies), however, both

systems are relatively cheap because no servicing is required. Furthermore, the bead separation systems do not require an operator as skilled as the one required for the FACS system. It should be noted, however, that magnetic separation is far more limited than FACS because immunomagnetic techniques can only separate cells into positive and negative populations and not, for example, into high and low expressors of a molecule, as is possible with FACS sorting ***(4)***. Furthermore, only cell surface molecules can be used as markers for magnetic separation of live cells, and not markers that distinguish cells by other means such as the expression of green fluorescent protein in transfected cells ***(5)***.

The purity of cell populations obtained by immunomagnetic sorting is dependent on producing a single-cell suspension, as any unwanted cell attached to a labeled cell will also be retained in the positively labeled fraction. Large clumps of cells may be removed from a suspension by passing the cell suspension through a 35–40-μm mesh; however, some cell doublets may still remain. In FACS sorting, the gating parameters may be set to sort only single cells, and thus a high level of purity is achieved, but at the expense of a reduction in cell numbers. In immunomagnetic sorting, cell doublets that contain only the desired phenotype can be retained while those that contain unwanted cells can be removed by using a double sorting method. Several approaches may be used to remove the unwanted cell types:

1. Label the "contaminating" cell type and remove these cells (including doublets containing only one of this phenotype). Retain the unlabeled cells and then positively sort the desired phenotype.
2. Positively sort the desired cell phenotype using removable beads. Remove these beads, resort using a marker of the unwanted cells, and then keep the final negative fraction and discard the positive cells.
3. Positively sort the desired cell type using MACS beads and then remove contaminating cells using Dynabeads (the small MACS beads are not sufficient to cause cells to be attracted to the Dynal magnet). In this case it is essential that the antibody on the Dynabeads does not recognize the antibody used for MACS sorting; otherwise, all the cells will become coated in Dynabeads.

Although the type of separations that can be carried out by immunomagnetic sorting are not as extensive as those by FACS sorting, it can prove a useful and relatively simple technique that can yield large numbers of highly purified cells.

2. Materials

1. Primary antibody: The correct dilution of the primary antibody should be determined by the user.
2. Biotinylated secondary antibody: A biotinylated secondary antibody directed against the primary antibody should be used if the only beads available for sorting are streptavidin coated, and the primary antibody is not already biotinylated.
3. Buffer: Phosphate-buffered saline (PBS), 0.5% w/v bovine serum albumin (BSA).

4. Magnetic beads: Dynabeads or MACS beads coated in appropriate primary or secondary antibodies or streptavidin.
5. Separation columns: Positive or negative selection columns are required for MACS separation, and the type of column should be chosen accordingly. The size of column to be used is determined by the number of cells to be separated.
6. Bead detachment: If Dynabeads are to be removed, DETACHaBEAD may be required, or if CELLection beads are used, DNase solution (supplied as part of a kit) will be required.

3. Methods

If the cells are going to be cultured, carry out all procedures in a laminar flow cabinet.

3.1. Cell Preparation

1. Prepare a single-cell suspension by standard methods depending on whether the cells are from tissues, blood, or cell cultures ***(6)*** (*see* **Note 1**).
2. If cell clumps are present, pass the cell suspension through a 35–40-μm mesh.
3. Count the cells using a hemacytometer (*see* **Note 2**).

3.2. MACS Separation

1. If using directly conjugated beads, then proceed to **step 5**. Suspend the cell pellet in a small volume (approx 200 μL/10^7 cells) of primary antibody diluted in buffer. The correct dilution should be determined by titration, with a likely concentration of antibody being 5–10 μg/mL.
2. Incubate the cell suspension at 4°C (on ice) for 40 min to 1 h with rocking or regular inversion to mix the cell suspension (*see* **Note 3**).
3. Wash cells with 5 mL of buffer and centrifuge at 300*g* for 5 min.
4. Repeat **step 3** twice (*see* **Note 4**).
5. Suspend cell pellet in appropriate amount of buffer according to bead manufacturer's instructions and add appropriate amount of beads (*see* **Note 5**). For most types of MACS microbead, resuspend cells in 80 μL of buffer plus 20 μL of beads per 10^7 cells (for fewer than 10^7 cells, still use 100 μL of total volume).
6. Mix and incubate for 15 min at 6–12°C (refrigerator) or 40 min at 4°C (on ice).
7. Wash cells with 5 mL of buffer and centrifuge at 300*g* for 5 min.
8. Resuspend cells in 500 μL of buffer.
9. Prepare a MACS column of appropriate size (*see* manufacturer's instructions). Columns are available that are designed specifically for positive or negative selection and should be chosen accordingly. Columns for positive selection are ready to use; those for negative selection should be attached via a three-way tap to a "flow regulator" (syringe needle) and a syringe filled with buffer.
10. Rinse the column with cold buffer (*see* **Note 6**).
11. Apply cells to the MACS column in a magnetic field.
12. Allow unlabeled cells to flow through the column and collect the effluent as the "negative fraction" (*see* **Note 7**).

13. If using a positive selection column, rinse the cells 3× by applying buffer to the column (within the magnetic field) using a volume appropriate to the column size (*see* manufacturer's instructions). For a negative selection column, turn the three-way tap to the "fill" position (i.e., open to the syringe and column but not the needle), remove the whole column assembly from the magnet, and back-flush the cells into the column with buffer from the syringe. Replace the column in the magnetic field and change the flow resistor to a higher gauge. Allow the cells to flow through once more and collect the effluent as the wash fraction. This fraction may contain both negative and weakly positive cells and is usually discarded.
14. Fill the column once more with buffer and elute the positive cells outside the magnetic field using the supplied plunger for positive selection columns or by attaching the syringe to the top of a negative selection column and removing the flow resistor.

3.3. Dynabead Separation

1. If using directly conjugated Dynabeads, proceed to **step 2** below. If using a primary antibody followed by secondary antibody-coated beads, follow **steps 1–4** of **Subheading 3.2.,** then proceed to **step 2** below.
2. Suspend a cell pellet in an appropriate amount of PBS–BSA according to bead manufacturer's instructions and add an appropriate amount of beads (*see* **Note 8**).
3. Mix and incubate for 15–30 min at 2–8°C with rocking or occasional inversion.
4. Add 5 mL of buffer to the cell suspension, mix gently, and then place the tube into the Dynal magnet and leave for 1 min, during which time the beads and any attached cells are drawn to one side of the tube.
5. Carefully aspirate off the buffer containing unlabeled cells, making sure that the beads and labeled cells are not disturbed, and retain this as the negative fraction.
6. Remove the tube from the magnetic field and repeat **steps 4** and **5** (*see* **Note 9**).
7. Suspend the beads and labeled cells in buffer and retain this as the positive fraction.
8. If the beads are to be detached from the positively selected cells, follow **steps 9–13** for removal with DETACHaBEAD (certain types of beads only) or **steps 14–16** where CELLection beads have been used.
9. Suspend the positively labeled cells in 100 μL of buffer (this will suffice for 10^6–10^7 cells).
10. Add 1 U (10 μL) of DETACHaBEAD and incubate at room temperature with tilting and rotation for 45–60 min (*see* **Note 10**).
11. Place the tube in the Dynal magnet and leave for at least 1 min.
12. Carefully aspirate off the buffer containing the cells and retain.
13. Resuspend the beads and repeat **steps 11–12** to release any trapped cells.
14. Where CELLection beads are to be removed, resuspend the rosetted cells in 200 μL of buffer prewarmed to 37°C. This is sufficient for up to 5×10^7 beads, that is, approx 10^7 cells.
15. Add 4 μL of DNase solution (provided by Dynal as part of the CELLection bead kit). This is sufficient for up to 10^8 Dynabeads.
16. Incubate at room temperature, with tilting, for 15 min.
17. Flush rosettes through a pipet several times.
18. Follow **steps 11–13** above.

4. Notes

1. Exposure of the cells to trypsin should be minimized to reduce cell damage. Overtrypsinized cells are particularly fragile and may be more easily damaged by the labeling procedure.
2. It can be helpful to assess the number of dead cells in the suspension by carrying out a trypan blue dye exclusion test. Both Dynabeads and MACS beads can stick to dead cells nonspecifically, and it may be worth removing the dead cells at this stage by density gradient centrifugation.
3. All solutions should be kept cold to avoid antibody internalization by the cells.
4. Any remaining free primary antibody must be completely removed or it may bind to the beads and hinder their attachment to the cells.
5. If the primary antibody is biotinylated and streptavidin-coated beads are to be used for separation, ensure that the buffer used is biotin free.
6. Passing the buffer through the column both precools it and may reduce nonspecific interactions of the cells with the column material.
7. A negative selection column may become blocked because of trapped air bubbles. These may be released by gently applying pressure to the side syringe.
8. Dynabeads are provided in a buffer containing azide which should be removed before use. The azide may be removed by placing the bead solution in the separation magnet, removing the bead-free buffer and resuspending the beads.
9. The purity of positively sorted cells can be improved by repeating this step up to 3× to release trapped cells.
10. Check an aliquot of cells microscopically to ensure that the beads have been removed. If beads remain, the cells can be incubated for longer, or more DETACHaBEAD added.

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