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Population Enrichment and Isolation with Magnetic Sorting

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14. ABSTRACT

Manual and semi-automated sorting methods (MACS) provide a high-throughput, less costly method for sample enrichment compared to fluorescence activated cell sorting (FACS). Magnetic sorting methods use commercially available magnetic beads with many surface modifications available for customizable sorting methods. In this note, we outline the methods for magnetic cell sorting using manual and benchtop magnets (MACS), as well as a micromagnetic sorter (MMS) that uses diposable, microfluidic cartridges. Along with magnetic sorting methods, we detail flow cytometry analysis techniques to quantify cell population enrichment for isolation of bacterial display library reagents.

15. SUBJECT TERMS

MACS, magnetic sorting, display library

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Foreword

The purpose of this report is to outline procedures and methods as a guide for magnetic sorting. The protocol presented has been established from a previously funded ICB 6.2 research project funded through the Army university-affiliated research center (UARC), the Institute for Collaborative Biotechnologies (University of California, Santa Barbara under grant DAAD19-03-D-0004), current mission work in synthetic recognition elements, and a current 6.1 research project funded by the Defense Threat Reduction Agency (DTRA, Grant no. BRCALL08-Per3-P-2-0028) for smart affinity reagent development. These methods can be used directly for magnetic sorting or can serve as a guideline for developing new magnetic sorting methods.

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Introduction

Magnetic sorting is a convenient, low-cost method used to isolate antibody alternatives (magnetic activated cell sorting [MACS]) and is often used to enrich cell populations prior to fluorescence activated cell sorting (FACS), due to the high-throughput achieved with MACS (*1*). There are a number of current magnetic sorting methods. Manual MACS uses a benchtop, rare-earth magnetic that is typically housed in plastic and can be purchased for different tube sizes. The autoMACS (Miltenyi Biotech) is semi-automated and is marketed for sorting against eukaryotic cells, including yeast. The autoMACS uses a disposable magnetic column for trapping cells labeled with magnetic beads. Recently, the U.S. Army Research Laboratory (ARL) transitioned a microfluidic magnetic sorter (MMS) from Cynvenio Biosystems during an ICB 6.2 project that was developed as a low-cost magnetic sorter for sorting against bacteria display libraries (*2*). The MMS uses disposable microfluidic cartridges, relying on a high-surface area trapping region for efficient cell capture. Besides being used for bacterial library sorting, the MMS can also be used for eukaryotic cell sorting and protein purification using magnetic beads.

Herein, we describe the procedures for employing magnetic sorting for bacterial display library enrichment, using either MMS or manual MACS. The following method was developed using a bacterial display library expressing a random 15-amino acid peptide on the outer membrane surface (*3*–*5*). This method has also been used, with minor modifications, to sort yeast display libraries: yeast are larger cells, which results in greater sample volumes for an equal number of cells, and sorting with yeast requires longer growth periods because of the slower doubling rate. For a review on autoMACS sorting with yeast libraries see Chao, et al. 2006 (*6*). This outlined procedure will be useful for researchers new to library sorting procedures using manual MACS or a semi-automated sorter, such as the MMS.

Materials and Methods

Bacterial Culture Growth Materials:

Luria Broth (LB) (Fisher Scientific) supplemented with 25 μg/ml Chloramphenicol (Sigma) is used for all bacteria growth cultures, typically 15 g/L, and autoclaved prior to use (LB-Cm^{25}). For surface display induction, a 4% w/v L-arabinose stock (2.0 g in 50 mL Sigma) is used to supplement growth media, while a 20% w/v D-glucose solution (10.0 g in 50 mL H_2O) is used to inhibit induction during overnight growth and recovery steps. Both the arabinose and glucose solutions are sterile-filtered prior to use. LB-Cm^{25} agar are made, in house, using LB and agar

combined in solution, autoclaved, and cooled to approximately 45 °C before the addition of chloramphenicol to final concentration of 25 μg/mL.

MMS and MACS Sorting Materials:

A PBS solution, phosphate buffered saline, pH 7.4, is used as the binding buffer and PBS containing Bovine serum albumin (BSA) at 0.5% w/v final concentration is used as the wash buffers. For this procedure, the magnetic beads being used are Dynabeads® MyOne Strepavidin T1 beads (Invitrogen). The T1 beads are recommended for protein, biopanning, and cell isolation, and are compatible with BSA. Other Dynabead variants include M-280, M-270, and C1, and each bead ranges in size and suggested application (Invitrogen). Magnetic beads of varying sizes or surface modifications are available from other suppliers (Thermo Fisher Scientific, Miltenyi, Biorad, and more). For target coupling to T1 streptavidin beads, a biotinylation step is necessary. Biotin labeling kits are available from many suppliers (Pierce, Invitrogen, Qiagen, etc.) for labeling proteins with biotin through free amine moieties on surface exposed lysine residues. Unreacted biotin can be removed by desalting columns, dialysis, or gel filtration, or other method recommended by the biotinylation kit manufacturer.

Flow Cytometer Analysis Materials:

Streptavidin-R-Phycoerythrin (SAPE) and Neutravidin-R-Phycoerythrin (NAPE) from Invitrogen are used to fluorescently label biotinylated targets already bound to the cell surface. SAPE and NAPE are typically used at a 1:200 dilution of SAPE/NAPE:PBSB, and have excitation wavelength of 480, 545, and 565 nm, and an emission wavelength of 578 nm.

Magnetic Sorting Methods:

Initial Sort (Round 1):

1. Obtain bacterial library (eCPX or other similar library) and amplify library by growing culture in LB Cm^{25} overnight and aliquoting at least $3x10^{11}$ cells/tube, and storing at –80 °C.
2. Add one frozen aliquot into 500 mL of LB-Cm^{25} (LB + 25μg/mL Chloramphenicol) and grow at 37 °C with shaking to an OD_{600} (optical density at 600 nm) of 0.55–0.70. This growth step typically requires 1 hr and 45 min.
3. Once grown to the correct OD_{600}, induce the library with final concentration of 0.04% Arabinose (5 mL of 4% Arabinose stock solution in 500 mL) and incubate for 35 min at 37 °C. Check OD_{600} after induction. At 1 OD_{600}, there are approximately$1x10^9$ cells/mL.
4. Centrifuge $2x10^{11}$ cells (table 1, cells for sorting Round 1) at 5000 x g for 20 min.
5. Remove supernatant and resuspend cells in 1.5 mL of PBS buffer. To resuspend pellet, gently swirl until dissolved; DO NOT VORTEX.

Library Depletion with Magnetic Beads (Negative Sort)

1. Resuspend cell pellet in 1 mL of 10^9 washed Strepavidin T1 beads.
    a. 10^9 beads is 100 µL of stock solution beads, wash by resuspending beads in 1 mL of PBS then centrifuging at 5000 x g for 5 min, and finally resuspending in 1 mL of PBS.
2. Incubate cell suspension for 15 min at room temperature with gentle rotation.
3. Depletion by Magnetic Sorting:
    a. MMS instrument…Run the MMS "1st negative selection" (Day 1-1st negative Selection on software) to remove any cells that bind to the beads alone.
    b. Manual MACS
        i. Using a microcentrifuge magnet separator, place the 1.5 mL microcentrifuge tube against the magnet.
        ii. After 5 min, remove media that contains unbound cells and wash beads with 1 mL of PBSB by inverting the tube 3–4 times in the absence of the magnet before returning the sample to the magnet for subsequent wash steps. Combine all wash samples with original unbound cell sample as streptavidin depleted library. After three washes, discard the magnetic beads.
4. Centrifuge the positive collection tube (containing the depleted library) at 5000 x g for 5 min, then remove supernatant and resuspend in 1 mL of PBS.

Positive Sorting Round 1 after Depletion

1. Transfer cells to a 2 mL centrifuge tube and incubate with biotinylated target at 4 °C with rotation for 45 min. The starting concentration for each target is typically 600 nM in PBS (table 1) but may be increased to a ~1 µM starting concentration if low enrichment is not seen after 2–3 rounds.

Table 1. Typical parameters for magnetic sorting with MMS or manual MACS using bacterial display libraries as detailed in the text.

	Round 1	Round 2	Round 3	Round 4
Cells for Sorting	10-times the library diversity (2×10^{11} for eCPX library)	5 - 10-times the depleted library diversity (at least 1×10^{8} cells for bacterial display)	5 - 10-times the depleted library diversity (at least 1×10^{8} cells for bacterial display)	5 - 10-times the depleted library diversity (at least 1×10^{8} cells for bacterial display)
T1 Magnetic Beads	100 µl	15 µl	8 µl	4 µl
Typical Target Concentration	600 nM	300 nM	150 nM	75 nM
Sample Volume **Mixing Buffer Vol.** **Wash Buffer Vol.**	2 mL, 3 mL, 8 mL	500 µL 800 µL 8 mL	500 µL 800 µL 8 mL	500 µL 800 µL 8 mL
Typical Plating Dilutions	1:10,000 1:100,000 1:1,000,000	1:100 1:1000 1:10,000	1:10 1:100 1:1000	1:2 1:10 1:100

2. Centrifuge cells at 5000 x g for 5 min then remove supernatant.
3. Resuspend cell pellet in 1 mL of 109 washed Streptavidin T1 beads. Approximate volume of sample is 2 mL with bead solution and cell pellet.
4. Incubate the cells at 4 °C for 30 min with rotation to allow binding between the beads and biotinylated target.
5. Magnetic Sorting (MMS or manual MACS)
    a. MMS
        i. Prepare PBSB wash buffers and mixing buffers (table 1).
        ii. Run the MMS "1st Round positive selection" (Day 1-1st Positive Selection on software) to sort the library.
        iii. When inserting the syringes onto the MMS card, ensure that a firm connection has been made without pressing down too hard. If leaking of the buffer solutions is seen above the MMS card after the sort has finished, the syringes may not have been secured well enough. If leaking occurs below the MMS card, back pressure

from the syringes being too tight may have caused a rupture in the fluidics membrane.

iv. After the sort has been completed, place both the positive and negative collection tubes against a magnet to ensure all of the magnetic beads have been retained in the positive collection tube.

1. If a significant amount of beads has not been recovered in the positive or negative sort, they may have been trapped on the MMS card, in which case the sort may need to be repeated with a new card.

2. Continue with post-sorting processing with positive sample.

b. Manual MACS

i. Using a multicentrifuge magnet separator, place the 1.5 mL microcentrifuge tube against the magnet.

ii. Sample washing (repeat 3 times). After 5 min, remove the solution and wash the beads with 1 mL of PBSB by inverting the tube 3–4 times in the absence of the magnet before returning the sample to the magnet for subsequent wash steps. After three washes, resuspend the beads with 1 mL PBSB for post-sorting processing.

<u>Post-sorting Process for all Sorting Rounds</u>

1. Warm 4 LB-Agar Cm^{25} plates for 2 h at 37 °C.
2. Remove a 10 μL sample from the positive collection tube and dilute in PBS. (See table for plating dilutions).
3. Plate 100 μL of each sample dilution and a 100 μL sample of buffer alone on LB-Cm^{25}-Agar plates.
4. Add the remaining positive sample to a 5 mL LB-Cm^{25} culture containing 0.2% glucose, final concentration, using a 20% stock glucose solution.

<u>Sorting after Overnight Growth (Round 2–4 sorting)</u>

1. Passage a fresh bacteria culture from overnight growth culture using 100 μL in 5 mL LB-Cm^{25} without glucose. *Glucose inhibits the induction with arabinose.
2. Grow cells at 37 °C for approximately 1 h and 45 min; begin checking the OD_{600} after 1 h.
3. Bacteria culture with 500 μL of 30% v/v Glycerol:H_2O stock (final glycerol concentration of 15%).

4. Once grown to OD_{600} of 0.55–0.7, induce the library with a final concentration of 0.04% arabinose and incubate for 35 min at 37 °C. Check OD_{600} at induction completion. At 1 OD_{600}, there are approximately$1x10^9$ cells/mL.

5. During induction, prepare flow cytometer labeling solutions (See Flow Cytometer Labeling section).

6. After induction, centrifuge cells for sorting (table 1, cells for sorting Round 2–4) at 5000 x g for 5 min.

7. *Optional*. Negative sorting with Streptavidin T1 beads in round 2.

    a. Wash 1x108 Streptavidin T1 beads in 1 mL PBSB, centrifuge beads at 5000 x g for 5 min, and resuspend in 50 μL PBS.

    b. Run MMS (Day 2, 1st Negative Selection) using 1.5 mL microcentrifuge tube to collect cells, instead of 15 mL Falcon tube.

8. Remove supernatant and resuspend cells in 50 μL of PBS buffer containing biotinylated target at the desired concentration (table 1, typical target concentration Round 2–4). To resuspend pellet, gently swirl until dissolved; *do not vortex.*

9. Incubate the cells with biotinylated target protein on ice for 45 min.

10. Wash magnetic beads (table 1, magnetic beads for Round 2–4) with 1 mL PBS, centrifuge at 5000 x g for 5 min, and resuspend beads in 50 μL PBS1. (100 μL of Streptavidin T1 beads is approximately 10^9 beads.)

11. Centrifuge sample at 5000 x g for 5 min, decant supernatant, and resuspend pellet in 50 μL solution of magnetic beads.

12. Incubate sample and magnetic beads on ice for 30 min.

13. Adjust sample volume to 500 μL with PBSB for sorting (See table 1, Sample Vol.).

14. Magnetic Sorting

    a. Prepare mixing buffer volume and wash buffer volume using PBSB, according to table 1.

    b. Run the MMS positive selection using Day 2 for round 2 sorting and Day 3 for round 3 and 4 sorting. Continue with post-sorting processing. (See Post Sorting Process Section).

    c. Manual MACS

        i. Using a multicentrifuge magnet separator, place the 1.5 mL microcentrifuge tube against the magnet. In rounds 2–4, 0.5 mL of PBSB is used for washing.

ii. In subsequent sorting rounds, it may be difficult to visualize the magnetic beads with the magnetic microcentrifuge tube holder. If this occurs, the wash steps should be performed by centrifugation (5000 x g for 5 min) instead of using manual MACS.

v. After three washes, resuspend the beads with 1 mL PBS for post-sorting processing.

15. Perform post-sorting processing.

Flow Cytometer Labeling

1. After induction, remove 5 μL of induced cells, and incubate with a 25 μL solutions of PBS containing 250 nM solution of biotinylated target, SAPE (positive control), NAPE (positive control), and 25 μL of PBS alone for negative control. Incubate the samples on ice for 45 min.
2. After 45 min of incubation, centrifuge the samples at 5000 x g for 5 min, decant the supernatant, and begin measuring the SAPE, NAPE, and cells alone (negative control) on the flow cytometer. For flow cytometry analysis, each pellet should be resuspended in 0.5–1.0 mL of BD FacsFlow®. The biotinylated target should be resuspended in a 25 μL, 250 nM solution of SAPE/NAPE for 30 min on ice for fluorescent labeling.
3. After the 30-min incubation, centrifuge the sample at 5000 x g for 30 min and measure the percent fluorescence of the cells labeled with the biotinylated target. This value indicates the population enrinchment in each round.

Results and Discussion

A typical magnetic sort shows positive population enrinchment with each round. In figure 1, the population enrichment results are shown using flow cytometry to evaluate the library after each sort. The percent positive population is displayed in green on each plot in figure 1, while the red populations are cells without PE on the surface. Most flow cytometers have excitation and emission filters, and optimized settings for PE fluorescence; therefore, PE is a common choice for flow cytometry. Alternatively, amine reactive fluorescent dyes or quantum dots can be directly coupled to the target for direct detection by flow cytometry to avoid non-specific binding of neutravidin or streptavidin (*7, 8*), or PE, which is a large, 240-kDa fluorescent protein from marine algae (*9, 10*).

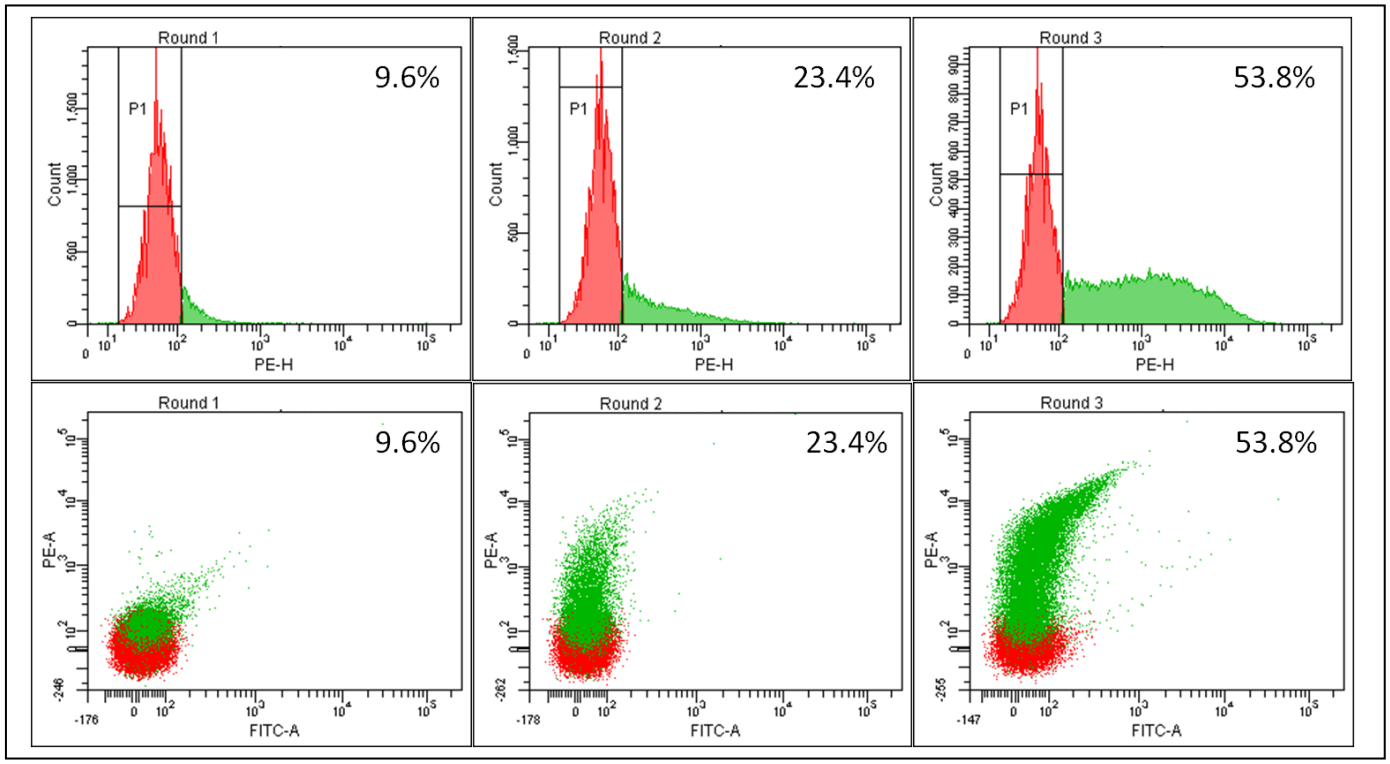

Figure 1. Typical enrinchment results for magnetic sorting using MMS. The increase in the PE signal (green) in each round (1-3, left to right) indicates an enriched positive population. The positive increase is noted on the x-axis (PE-H) in the histograms in the top panel and along the y-axis in the dot plot in the bottom panel. The red signal in each plot is the background cell fluorescence measured in the PE emission channel. Either a histogram of PE-H vs. count or a dot-plot with FITC-A vs. PE-A is typical to show population enrichment by flow cytometry.

After one round of sorting, the increase in the PE signal indicates that 9.6% of the cells measured are positive for the sorted target. The number of positive cells increases from 9.6% to 23.4% to 53.8% after round 3. The total enriched population increase per round of sorting is a strong indication that the sorting was successful, and a high affinity binder is likely. The sorting is concluded when the library enrichment does not change between each round. For bacterial display, this can occur after 3 rounds of sorting, while most sorts with the MMS do require 4 rounds.

The greatest advantage of magnetic sorting is that populations of $10^9 – 10^{10}$ cells or greater can be sorted, compared to an upper limit of 10^8 cells per sort using FACS (*11*). As noted in table 1, it is advisable to initiate cell sorting with a 10-times oversampling of the library; therefore, FACS would be limited to libraries that have a diversity of 10^7 or less. This limitation of FACS is problematic for sorting libraries that can have diversities of 10^{10} or greater such as bacterial, mRNA, and ribosomal display (*11, 12*). Using a microfluidic-based sorter such as MMS extends the sorting throughput to beyond 10^{10} cells per sort, which is typically the limit of magnetic sorting.

Conclusion

Magnetic sorting using MMS or manual MACS is currently the most widely used method for library depletion for libraries with diversities greater than 10^7 unique members. Magnetic sorting is a low-cost alternative to FACS, since library depletion can be accomplished using a strong magnet on the benchtop. The procedures and methods presented in this report can serve as a guide to design experiments for library depletion, including methods to evaluate library enrichment using flow cytometry.

References

1. Siegel, R. W.; Coleman, J. R.; Miller, K. D.; Feldhaus, M. J. High Efficiency Recovery and Epitope-specific Sorting of an scFv Yeast Display Library. *J Immunol Methods* **2004**, *286* (1-2), 141–53.

2. Stratis-Cullum, D. N.; Kogot, J. M.; Pellegrino, P. M. *Rapid Peptide Reagent Isolation in a Disposable Microfluidic Cartridge*; Army Research Laboratory: 2010; p 19.

3. Daugherty, P. S. Protein Engineering with Bacterial Display. *Curr. Opin. Struct. Biol.* **2007**, *17*, 474–480.

4. Rice, J. J.; Schohn, A.; Bessette, P. H.; Boulware, K. T.; Daugherty, P. S. Bacterial Display Using Circularly Permuted Outer Membrane Protein OmpX Yields High Affinity Peptide Ligands. *Protein Sci* **2006**, *15* (4), 825–36.

5. Bessette, P. H.; Rice, J. J.; Daugherty, P. S. Rapid Isolation of High-affinity Protein Binding Peptides Using Bacterial Display. *Protein Eng Des Sel* **2004**, *17* (10), 731–9.

6. Chao, G.; Lau, W. L.; Hackel, B. J.; Sazinsky, S. L.; Lippow, S. M.; Wittrup, K. D. Isolating and Engineering Human Antibodies Using Yeast Surface Display. *Nat Protoc* **2006**, *1* (2), 755–68.

7. Alon, R.; Bayer, E. A.; Wilchek, M. Cell-adhesive Properties of Atreptavidin are Mediated by the Exposure of an RGD-like RYD Site. *Eur J Cell Biol* **1992**, *58* (2), 271–9.

8. Alon, R.; Bayer, E. A.; Wilchek, M. Streptavidin Contains an RYD Sequence Which Mimics the RGD Receptor Domain of Fibronectin. *Biochem Biophys Res Commun* **1990**, *170* (3), 1236–41.

9. Parks, D. R.; Herzenberg, L. A. Fluorescence-activated Cell Sorting: Theory, Experimental Optimization, and Applications in Lymphoid Cell Biology. *Methods Enzymol* **1984**, *108*, 197–241.

10. Kronick, M. N.; Grossman, P. D., Immunoassay Techniques with Fluorescent Phycobiliprotein Conjugates. *Clin Chem* **1983**, *29* (9), 1582–6.

11. Park, S. J.; Cochran, J. R. *Protein Engineering and Design*; CRC Press: Boca Raton, 2010; p 23–50.

12. Lipovsek, D.; Pluckthun, A. In-vitro Protein Evolution by Ribosome Display and mRNA Display. *J Immunol Methods* **2004**, *290* (1-2), 51–67.

List of Symbols, Abbreviations, and Acronyms

ARL	U.S. Army Research Laboratory
BSA	bovine serum albumin
Cm^{25}	Chloramphenicol at 25 μg/mL
LB	Luria Broth
FACS	fluorescence activated cell sorting
MACS	magnetic activated cell sorting
MMS	microfluidic magnetic sorter
PBSB	phosphate buffered saline with 0.5% BSA
SAPE	Streptavidin-R-phycoerythrin
NAPE	Neutravidin-R-phycoerythrin

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