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Rapid and Efficient Selection of Yeast Displaying a Target Protein Using Thermo-responsive Magnetic Nanoparticles

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Magnetic separation provides a relatively quick and easy-to-use method for cell isolation and protein purification. We have developed a rapid and efficient procedure to isolate yeast cells displaying a target polypeptide, namely, the *Staphylococcus aureus* ZZ domain, which serves as s model for protein interactions and can bind immunoglobulin G (IgG). We optimized selection of ZZ-displaying yeast cells using thermoresponsive magnetic nanoparticles. A model library was prepared by mixing various proportions of target yeast displaying the ZZ domain with control cells. Target cells in the model library that bound to the ZZ-specific binding partner, biotinylated IgG, were selected with biotinylated thermoresponsive magnetic nanoparticles using the biotin-avidin sandwich system. We determined ZZ expression levels and optimized the concentrations of both magnetic nanoparticles and avidin for efficient selection of target cells. After optimization, we successfully enriched the target cell population 4700-fold in a single round of selection. Moreover, only two rounds of selection were required to enrich the target cell population from 0.001% to nearly 100%. Our results suggest that magnetic separation will be useful for efficient exploration of novel protein-protein interactions and rapid isolation of biomolecules with novel functions.

Introduction

Directed evolution and screening of combinatorial libraries are effective methods for exploration and engineering of new protein functions and properties. Protein display on the surface of various microorganisms is a commonly used and well-established approach (*1*−*3*). Among cellular display systems, yeast display systems are particularly attractive for screening due to ease of genetic engineering and linkage of phenotype to genotype. Yeast display systems are also suitable for screening of mammalian proteins, as yeast protein folding and secretory machineries are similar to those of mammals. Indeed, utility of this approach is evident in a variety of successful applications of the technology, such as engineering of antibodies and recombinant vaccines (*2, 4*).

Selection of target cells in pools is a critical step in screening. Flow cytometric sorting is one of the most reliable and quantitative techniques for high-throughput screening. However, a flow cytometer is rather expensive and its use is not equally feasible for all researchers. Compared with a flow cytometer, magnetic separation is a more convenient and affordable tool for cell sorting that can also be used for protein purification (*5*−*7*). In cell sorting via magnetic separation, cells bound to specific ligands on the surface of the magnet beads can be separated from unbound cells with a magnet. At least in principle, target cell isolation with magnet beads should relatively quick and easy, even when applied to a large library.

We previously reported results of separation of target yeast cells from large pools of cells using biotinylated thermoresponsive magnetic nanoparticles (TMLB) (*8*). The advantages of TMLB are that they can disperse completely and that they have sufficient surface area for target adsorption because the diameter of TMLB is less than 1 μm. In addition, the thermoresponsive properties that reversibly transition between flocculation and dispersion with a low critical solution temperature (LCST) of 34 °C enable easy magnetic separation by flocculation (*9, 10*). However, utility of the method would benefit greatly from optimization of the conditions for yeast cell separation using magnetic nanoparticles.

In the work reported here, we optimized magnetic cell isolation from a model library toward the goal of rapid and efficient selection of target cells. As in our previous study (*8*), magnetic separation was carried out using the following materials and procedures. As a model protein for display on the yeast cell surface, we used the ZZ domain from *Staphylococcus aureus* (*11*−*13*). The 3′-half of the region encoding 320 amino acids of α-agglutinin was used as anchor protein for yeast surface display system with the secretion signal sequence of glucoamylase derived from *Rhizopus oryzae*. Biotinylated IgG is a specific ligand of the ZZ domain and thus was used for capture of target cells. TMLB was bound to biotinylated IgG via a specific interaction based on the biotin−avidin sandwich

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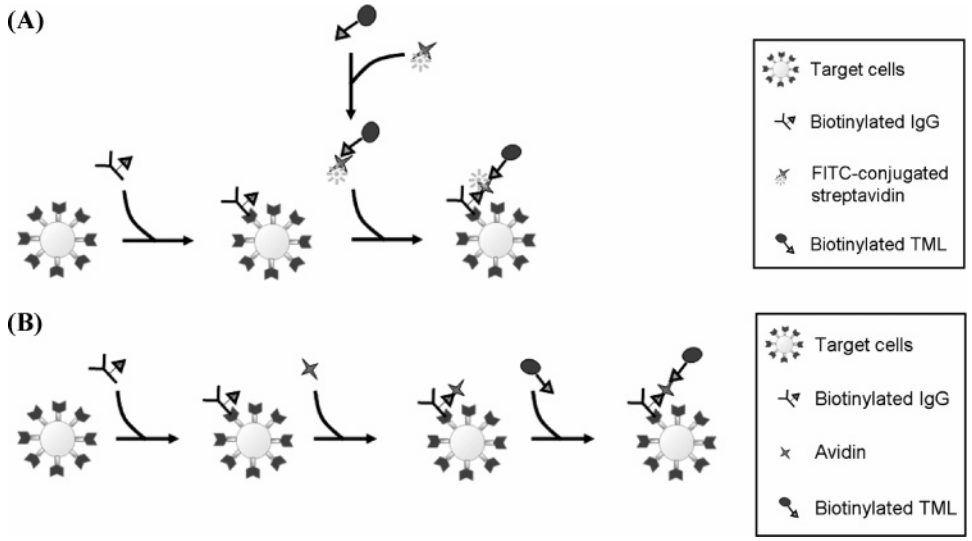

Figure 1. Schematic representation of ZZ-IgG binding and the magnetic nanoparticle approach to target cell isolation. (A) Schematic illustration of specific binding between displayed proteins (ZZ), biotinylated ligands (IgG) and thermoresponsive magnetic nanoparticles with FITC-conjugated streptavidin. (B) Schematic illustration of magnetic nanoparticle-target yeast cell complexes and magnetic separation.

technique. This method can be used to display a wide variety of proteins, as other biotinylated ligands can be substituted for biotinylated IgG. We demonstrate that optimization of expression of the displayed protein and of TMLB or avidin concentrations can result in up to 4700-fold enrichment with half the number of rounds of selection we reported previously (*8*). Our results should make possible the development of further applications that use magnetic separation for efficient exploration of novel protein−protein interactions and rapid isolation of biomolecules with novel functions.

Materials and Methods

Strains and Plasmids. The yeast strain used for cell-surface display was *Saccharomyces cerevisiae* BY4741 (*14*). The plasmid pGUZZ was used for cell-surface display of the ZZ domain using the secretion signal sequence of glucoamylase derived from *Rhizopus oryzae* and the 3′-half of the region encoding 320 amino acids of α-agglutinin under the control of *GAL1* promoter as described previously (*15*, *16*). The plasmid pESC-URA (Stratagene, La Jolla, CA) was used as a control.

Yeast Transformation and Cultivation. Each plasmid was introduced into *S. cerevisiae* BY4741 using the lithium acetate method (*17*). Yeasts for feeding were grown in 5 mL of SDC media containing 0.67% yeast nitrogen base without amino acids (YNB; Becton, Dickinson and Company, Franklin Lakes, NJ), 2% glucose and 2% casamino acids (w/v). Cells were transferred to 10 mL of SGC media containing 0.67% YNB, 2% galactose and 2% casamino acids to induce expression of the ZZ domain and cultured to an initial optical density of 0.03 at 600 nm (initial $OD_{600} = 0.03$). The cells were subsequently cultivated for 16 h prior to harvesting for further analysis or selection.

Flow Cytometric Analysis. The fluorescence intensity of yeast cells was measured on a FACSCalibur equipped with a 488-nm air-cooled argon laser (Becton Dickinson and Company, Franklin Lakes, NJ), and the data were analyzed using CELLQuest software (Becton Dickinson and Company, Franklin Lakes, NJ). Parameters were as follows: the amplifiers were set in linear mode for forward scattering (FSC) and in logarithmic mode for the green fluorescence detector (FL1, a 530/30-nm band-pass filter) and the orange fluorescence detector (FL2, a 585/21-nm band-pass filter); the amplifier gain was set at 1.00 for FSC; the detector voltage was set to E00 for FSC, to 600 V for FL1 and to 500 V for FL2; and the FSC threshold was set at 52. Geometric mean fluorescence was determined using the green fluorescence signal collected from 10,000 cells.

Immunofluorescence Detection of ZZ Domain Polypeptide on Yeast Cell Surface. Yeast cells were cultivated in SGC media for 16 h. The cells were then harvested and washed with phosphate-buffered saline (PBS, 50 mM phosphate, 150 mM sodium chloride, pH 7.4). The cells were resuspended in 100 μL of PBS to an optical density of 10 at 600 nm ($OD_{600} = 10$). Next, cells were mixed with 10 μg/mL anti-protein A antibodies labeled with FITC (Immune System I.M.S AB, Uppsala, Sweden). After 30 min of incubation at room temperature, the cells were washed with PBS. The cells were then analyzed with a flow cytometer (FCM) as described above.

Complex Formation between Thermoresponsive Magnetic Nanoparticles and ZZ-Displaying Cells. TMLB (Thermo-Max LB Biotin; Magnabeat, Chiba, Japan) coated with fluorescein isothiocyanate (FITC) conjugated streptavidin-coated were prepared as outlined in Figure 1A. Streptavidin-FITC (Zymed laboratories, South San Francisco, CA) was added to 0.2% TMLB solution (% w/v; final concentration of streptavidin, 100 μg/mL). After 15 min of gentle stirring at room temperature, the TMLB-streptavidin-FITC complex was collected with a magnet and the supernatant was removed to eliminate excess avidin. The TMLB-streptavidin-FITC complex was then washed three times with PBS. In parallel, 1 μL of 1 mg/mL biotinylated rabbit IgG (Vector laboratories, Burlingame, CA) was added to 100 μL of yeast cell suspension ($OD_{600} = 10$). After 30 min of gentle stirring at room temperature, yeast cells were washed with PBS. The TMLB-streptavidin-FITC complex was combined with the IgG-treated yeast cells, and the mixture was stirred for an additional 30 min. The yeast cells were harvested by centrifugation at 4,000 g for 5 min, then washed and resuspended with PBS. The fluorescence intensity of yeast cells was measured and normalized on FCM as described above. Normalized fluorescence intensity was determined by subtracting the geometric mean fluorescence of unstained cells from that of stained cells.

Preparation of Model Libraries. Model libraries were prepared by mixing various ratios of ZZ-displaying cells (target cells; BY4741/pGUZZ) with control cells that do not display

ZZ (BY4741/pESC-URA). The number of cells was determined by measuring the optical density at 600 nm (OD_{600}).

Isolation of Target Cells from Model Library by Magnetic Separation. A schematic illustration of target cell isolation from the library is shown in Figure 1B. The model libraries were cultivated in 10 mL of SGC media for 16 h (initial OD_{600} = 0.03). Cells were harvested and washed with PBS and resuspended in 100 μL of PBS containing 10% BSA (% w/v; New England Biolabs, Ipswich, MA) to give an optical density of 10 at 600 nm (OD_{600} = 10). Then, 1 μL of 1 mg/mL biotinylated rabbit IgG was added to 100 μL of cell suspension. After 30 min of gentle stirring at room temperature, 1 μL of 10 mg/mL avidin solution (Calzyme, Sun Luis Obispo, CA) was added and the suspension was stirred for 30 min. In order to remove excess avidin, cells were centrifuged and washed three times with PBS containing 10% BSA. After washing, 100 μL of 0.2% TMLB solution was added and the mixture was gently stirred at room temperature for 15 min. The solution was heated to 42 °C to flocculate the TMLB. The TMLB-yeast cell complexes were then collected with a magnet. After removing the supernatant, the TMLB-cell complexes were washed three times with PBS containing 10% BSA. Each of the collected cell complexes was resuspended in 100 μL of PBS and the suspensions were added to 5 mL of SDC media containing 100 μg/mL ampicillin (to prevent contamination during subsequent cultivation). The yeast cells were cultivated for approximately 16−18 h, until the OD_{600} value exceeded 1.0. Then, yeast cells were transferred to 10 mL of SGC media (initial OD_{600} = 0.03) and cultured for 16 h. The population of the target cells in the mixed cell population was analyzed as described below and these cells were also used in subsequent selection rounds.

Evaluation of Enrichment of Target Cells after Magnetic Separation. After each round of selection, immunofluorescence staining using anti-protein A antibodies labeled with FITC (Immune System I.M.S AB, Uppsala, Sweden) was performed as described above, and the proportion of target cells (ZZ-displaying cells) was evaluated using a calibration curve method as follows. Six model libraries were prepared, each with a different percentage of target cells (0.001%, 0.01%, 0.1%, 1%, 10% and 100%). The total number of cells was evaluated by determining the optical density at 600 nm (OD_{600}). The libraries were first grown in SDC media and then in SGC media to induce expression of the ZZ-containing polypeptide. After staining with anti-protein A antibody labeled with FITC, the population of ZZ-displaying cells was analyzed with FCM. Results from three independent experiments were then combined. Finally, the proportion of fluorescent cells was plotted versus ZZ-displaying cells in the total library population.

Results

Formation of Complex between Magnetic Nanoparticles and ZZ-Displaying Cells. First, to verify display of the ZZ domain on the yeast cell surface, yeast cells were stained with FITC-conjugated antibody and analyzed with FCM (Figure 2). Fluorescence of ZZ-expressing cells was greater than that of control cells, suggesting that the ZZ domain is efficiently displayed on the yeast cell surface.

To determine if the ZZ-displaying cells form complexes with TMLB via specific ligand-binding, we used the ZZ domain and IgG a model interaction pair. A schematic diagram of our approach is illustrated in Figure 1A. A biotinylated ligand (in this study, biotinylated IgG) interacts with the displayed protein (in this study, the ZZ) through specific interaction between the two polypeptides. Next, the TMLB-streptavidin-FITC complex

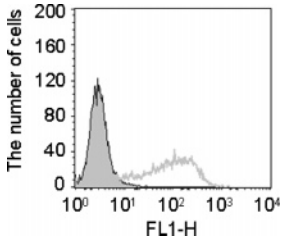

Figure 2. Flow cytometric analysis of ZZ-displaying cells. Closed histogram plot represents the control strain BY4741/pESC-URA, and open histogram plot represents the strain BY4741/pGUZZ. Vertical axis indicates number of cells (counts), and horizontal axis indicates green fluorescence intensity (FL1-H).

binds to the biotinylated ligand displayed on cell, via specific interaction between biotin and avidin. To detect complex formation, FITC fluorescence was determined by flow cytometry (FCM).

In the absence of biotinylated IgG, little or no fluorescence signal was detected (Figure 3A). By contrast, in the presence of biotinylated IgG, the fluorescence intensity of target cells harboring the plasmid for extracellular display of the ZZ domain was higher than that of control cells with the plasmid pESC-URA, which lacks the ZZ domain sequences.

Optimizing TMLB Concentration for Maximum Adsorption to ZZ-Displaying Cells. We next estimated the TMLB concentration able to saturate adsorption of the TMLB-streptavidin-FITC complexes (Figure 3B). The adsorption of the TMLB-streptavidin-FITC complex to the biotinylated-IgG-ZZ-cells was saturated at 0.1% TMLB (w/v; 100 μg TMLB per 1 $\times$ 10^7 yeast cells).

Separation of ZZ-Displaying Cells from a Model Library. Model libraries containing 0.1−0.001% ZZ-displaying target cells were prepared by mixing target and control cells. A schematic diagram of the magnetic separation is illustrated in Figure 1B. For the library approach, we first carried out magnetic separation under the following conditions: 10 μg/mL biotinylated IgG, 100 μg/mL avidin, 0.2% TMLB against 1×10^7 yeast cells.

Efficient separation of target cells from non-expressing cells was achieved after two to four rounds of selection (Figure 4). We also verified the target cell population after final selection round by colony PCR. The results show that all of randomly selected colonies have ZZ-displaying plasmid, suggesting complete enrichment from each model library (data not shown). Moreover, the population of target cells after a single selection round increased from a starting percent target cells of 0.001% to 0.085%, which means we achieved 85-fold enrichment in the first round of selection.

Optimization of Selection Conditions. To find conditions for more efficient selection, we tested the effect of variation of the avidin/TMLB concentration on efficiency of target cell selection. Magnetic separations were performed at 100 μL scale with each reaction including 1×10^7 yeast in which 0.001% of cells are target cells.

Initially, we tested the influence of TMLB concentration on selection efficiency (Figure 5A). Decreasing the TMLB concentration led to a concurrent increase in the efficiency of target cell selection, specifically, and target cell population went from 0.001% to 0.87% after single selection round, or 870-fold enrichment, when we used 0.04% TMLB. Subsequently, we tested the effect of changing the avidin concentration on selection efficiency when the TMLB concentration was 0.04% (Figure 5B). As expected, selection efficiency increased with a decreasing avidin concentration. For 0.1 μg/mL avidin, the

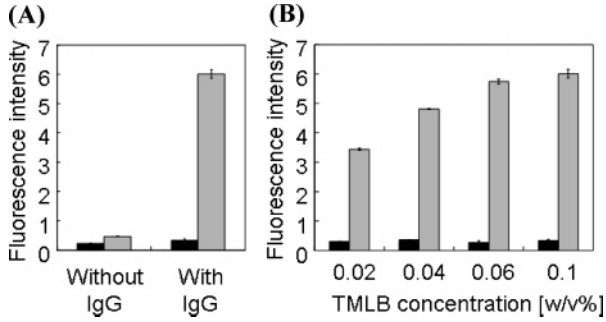

Figure 3. Adsorption of thermo-responsive magnetic nanoparticles on the yeast cell surface. Bars indicate the ZZ-displaying cells (gray bars) or control cells (black bars). (A) Fluorescence intensity after addition of the TMLB-streptavidin-FITC complex to yeast cells. (B) Fluorescence intensity under various concentrations of TMLB-streptavidin-FITC complex. Standard errors of three independent experiments are also represented.

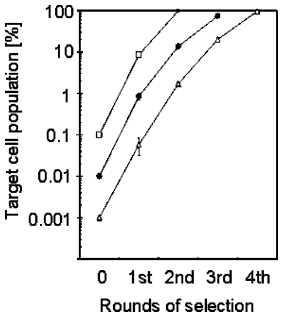

Figure 4. Enrichment of target cells after several rounds of selection. Selection conditions were as follows: 10 μg/mL biotinylated IgG, 100 μg/mL avidin, 0.2% (w/v) TMLB in the presence of yeast cells at an OD_{600} of 10 (1×10^7 yeast cells). The initial concentrations of target cells in the model libraries were indicated as follows: (□) 0.1%; (●) 0.01%; (△) 0.001%. Standard errors of three independent experiments are also represented.

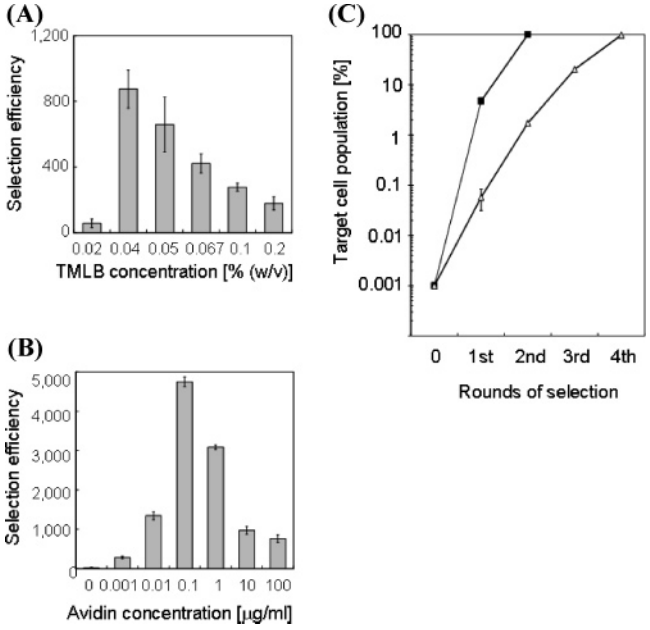

Figure 5. Optimization of the selection efficiency using a model library containing 0.001% target cells. We define selection efficiency as the population of target cells after selection divided by the population of target cells before selection (i.e., 0.001%). (A) Various concentrations of biotinylated magnetic nanoparticles. (B) Various concentrations of avidin. Standard errors of three independent experiments are also represented. (C) Isolation of target cells using an optimized protocol: (△) initial protocol; (■) optimized protocol. The optimal condition was as follows: 10 μg/mL biotinylated IgG, 0.1 μg/mL avidin, 0.04% TMLB in the presence of yeast cells at an OD_{600} of 10 (1×10^7 yeast cells). Standard errors of three independent experiments are also represented.

population of target cells was increased dramatically, from 0.001% to 4.7%, or 4700-fold enrichment. On the basis of these assays, we found that the optimal conditions are as follows: 0.04% TMLB and 0.1 μg/mL avidin.

Encouraged by these findings, we next asked how many rounds of selection are required to enriched the target cells to the level of >99% of the total cell population from a model library in which target cells represent 0.001% of the total cell population. Under optimized conditions (Figure 5C), we found that only two rounds of selection were necessary to reach >99% of the total cell population. These results indicate that with the optimized conditions, enrichment to this level takes about half the time we reported previously (*8*).

Discussion

The aim of this study was to establish a practical method for magnetic separation using thermoresponsive magnetic nanoparticles. Magnetic separation appears to be a promising method for use in directed evolution and screening studies based on combinatorial libraries. Moreover, magnetic separation is useful not just for cell isolation but also for protein selection and purification. Recently, magnetic separation attracted much attention as combining the magnetic separation method with yeast cell surface display has provided a rapid, efficient and reliable technology for protein evolution. Although several strategies for magnetic separation using yeast display systems have been reported (*5*, *8*, *18*−*20*), several problems with the method had persisted, including low separation efficiency and the requirement for several rounds of selection and growth of cells in order to enrich the target cell population. Therefore, we attempted to learn what factors are important to achieve highly efficient selection of target cells using only magnetic separation.

In this study, we used the ZZ domain, which has the ability to bind to the Fc region of rabbit IgG, as a model interaction protein. This was at least in part because use of the ZZ domain in cell-surface display has been well studied (*3*, *11*). Previously, we demonstrated the feasibility of affinity selection by flow cytometric sorting using the ZZ-displaying yeast cells (*15*). Moreover, the association constant of the ZZ domain displayed on the yeast cell surface for rabbit IgG has been estimated to be 2.1×10^8 M^{-1} (*15*), almost the same as that of the purified ZZ domain, 6.8×10^8 M^{-1} (*21*). Therefore, ZZ domain and IgG were preferred as a model interaction pair to investigate selection efficiency using magnetic separation. To expand the versatility of magnetic separation, we used biotinylated IgG as a model ligand. Using biotinylated thermoresponsive magnetic nanoparticles and the biotin−avidin sandwich system, target cells were efficiently isolated from a large library (Figure 1). One advantage of this method is that biotinylated beads can be used with a wide variety of displayed proteins and biotinylated ligands.

In a previous study, we developed a method for rapid and highly efficient selection of target cells by flow cytometric cell sorting. In that study, we also found that a critical factor for efficient selection is the level of expression of displayed protein (*15*). According to previous report, we used the *GAL1* promoter to enhance the level of expression of the ZZ domain polypeptide.

It has also been reported that in ZZ-displaying cells in which ZZ is under the control of the *GAL1* promoter, ZZ was expressed at high levels at an early stage of culture and thus allowed for rapid recovery of target cells by FCM sorting with few selection rounds (*15*). Therefore, we also examined the effect of protein expression levels on the efficiency of selection of target cells. We found that the ZZ domain was successfully displayed on yeast cells and formed complexes with TMLB via the ZZ-IgG interaction (Figure 3A). Then, we were able to find an optimal concentration of TMLB for maximum binding capacity to the ZZ-displaying cells (Figure 3B; 100 μg of TMLB per 1×10^7 yeast cells). In spite of an increase in ZZ domain expression level, however, selection of target cells from the model library by magnetic selection was only slightly improved (85-fold; Figure 4) as compared with a previous study (*8*). That suggested to us that factors affecting cell isolation were different for magnetic separation than for FCM.

Because the level of expression of the displayed protein did not appear to be an important factor for selection in magnetic separation, we suspected that nonspecific binding caused low selection efficiency in our initial experiments. As shown in Figure 3A, background fluorescence was recognizable in either the presence or absence of IgG for both control cells and ZZ-displaying cells. Therefore, to select target cells with high efficiency, we determined the effect of modifying the TMLB and avidin concentrations during magnetic separation. As shown in Figure 5, the efficiency of target cell selection after magnetic separation increased with decreasing concentrations of TMLB; indeed, the ratio of target cells to control cells was enriched 870-fold. Encouraged by these results, we tested the effects of modulating avidin concentrations in the presence of the optimized concentration of TMLB (namely, 0.04%). As expected, target cells were enriched, in this case up to 4700-fold higher than the value obtained using our initial conditions (Figure 5B). In Figure 5A and B, extreme decrease of the selection efficiency was observed under lower concentration of avidin or TMLB than the optimized concentration. These results may have been caused by defection of the magnetic force given for a cell under extremely low concentration of avidin or TMLB. Thus, optimization of TMLB and avidin concentrations with retaining its magnetic separation ability is crucial for effective selection of target cells using magnetic separation, which may suggest that an excess of TMLB or avidin results in nonspecific binding to yeast cell-surface.

Finally, we tested the number of rounds of selection necessary to enrich target cells up to nearly 100% of the cell population starting with a library in which target cells are just 0.001%. Using the optimized conditions, we successfully achieved highly efficient recovery of target cells after just two rounds of selection (Figure 5C).

Conclusion

In this work, we were able to demonstrate the feasibility of rapid and highly efficient selection of target cells using thermoresponsive magnetic nanoparticles. Optimization of TMLB and avidin concentration led to efficient enrichment and facilitated selection and isolation of target cells via magnetic separation. This approach may be applicable to other magnetic separation methods and we expect that use of magnetic separation will prove effective for directed evolution-type studies based on combinatorial library screening.

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