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Functional enrichment by direct plasmid recovery after Fluorescence Activated Cell Sorting

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Abstract

Iterative screening of expressed protein libraries using fluorescence-activated cell sorting (FACS) typically involves culturing the pooled clones after each sort. In these experiments, if cell viability is compromised by the sort conditions and/or expression of the target protein(s), rescue PCR provides an alternative to culturing but requires re-cloning and can introduce amplification bias. We have optimized a simple protocol, using commercially available reagents, to directly recover plasmid DNA from sorted cells, for subsequent transformation. We tested our protocol with two different screening systems in which less than 10% of sorted cells survive culturing and demonstrate that >60% of the sorted cell population was recovered.

Keywords

Plasmid recovery; library screening; toxicity

Body

Fluorescence-activated cell sorting (FACS) is a powerful high-throughput screening tool for protein engineering, provided a protein's function of interest can be coupled to and correlated with cell fluorescence. Screening of libraries for proteins with improved traits is typically accomplished by expression in a suitable microbial host and iterative sorting, pooling and culturing the sorted clones (1, 2). Unfortunately, enhancements in the protein's function of interest (*e.g.* enzymatic activity) and/or cell fluorescence (*e.g.* fluorescent protein expression) may compromise cell viability or growth (3, 4). Furthermore, some whole-cell protein assays require the cells to be permeabilized and thus rendered unviable (5). In such cases, "rescue" PCR is used to amplify the corresponding genes of the sorted cells. Apart from the subsequent re-cloning being time-consuming and laborious, PCR has potential to alter the frequency distribution of DNA sequences by amplification bias and homologous

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Author contributions

B.R and C.S.F performed the experiments for the chymotrypsin and the AraC system respectively. All authors designed the research and wrote the manuscript.

Competing Interests

The authors have no competing interests to report.

recombination (6). Alternate methods that allow for the direct recovery of plasmid DNA from sorted cells with high efficiency enable considerable savings in time and cost, while potentially preserving the genetic diversity and fitness improvements being sought.

Two key requisites of any method used for recovery during iterative library screening are: maximal retention of genetic diversity for subsequent sorting, and high yield of clones displaying the desired phenotype. Recently, a method to directly recover high-copy plasmid (~1000 copies/cell) from small numbers of cells (10^3–10^5) has been reported, but its effectiveness after FACS especially for low- and medium-copy plasmids is unknown (7). In the present study, we have quantitatively compared a direct plasmid recovery method with the more traditional method of culturing sorted cells following FACS, focusing on maximizing the yield of the sub-populations expressing the desired phenotype. We demonstrate enrichment and recovery using two disparate protein systems: *E. coli* MC1061 cells expressing the cytotoxic protease chymotrypsin, and *E. coli* HF19 cells expressing variants of the AraC regulatory protein that activate expression of GFP (8, 9). In both systems, direct plasmid recovery was found to be superior in comparison to recovery by culturing sorted cells.

As a first step, we evaluated three different lysis conditions for the cells expressing chymotrypsin B: i) 1X Bugbuster (EMD Millipore, MA) with 1 mg/mL lysozyme in 50 mM Tris-HCl pH 7.5 (buffer 1); ii) 100 mM EDTA and 1% Triton-X in 50 mM Tris-HCl pH 7.5 (buffer 2); and iii) 1X lysis buffer (buffer 3) for plasmid miniprep (Zymo research, CA). Buffer 3 contains 0.1 M NaOH, 0.7% SDS, 0.7 mM EDTA, and 1% isopropanol (10). For evaluating buffers 1 and 2, *E. coli* MC1061 cells (N_0 = 50,000 in ~200 μL) harboring plasmid pBAD_AChy_700 (p15A origin, 15–20 copies/cell) were sorted and incubated with an equal volume of 2X lysis buffer at 25 °C for 15 min (8, 11). Plasmids were purified using Zymo DNA clean and concentrator kit (Zymo research, CA). For buffer 3, an equal number of cells were sorted and the protocol prescribed for Zyppy plasmid miniprep kit (Zymo research) was followed using the DNA binding columns (Cat. No. D4004, Zymo research, CA). Plasmid DNA was eluted with 10 μL of Zyppy elution buffer (10 mM Tris, 1 mM EDTA, adjusted to pH 8), and 2 μL of eluent was used to transform electrocompetent *E. coli* MC1061 cells (transformation efficiency > 10^9 cfu/μg) (12). The total number of transformants (N_1) was estimated by plating transformation dilutions onto LB-agar plates supplemented with chloramphenicol (LB-Cm plates). The recovery efficiency, defined as the ratio of total number of transformants obtained to total number of sorted cells used for plasmid recovery (N_1/N_0), was highest when using alkaline lysis buffer 3 (44 ± 24 %) in comparison to buffer 1 (16 ± 13%) or buffer 2 (4 ± 2%). A step-by-step experimental protocol to perform DNA isolation using buffer 3 is available as a supplementary document online.

A library of chymotrypsin B (chylib1) variants containing a C-terminal FLAG tag was constructed using overlap-extension PCR and cloned into vector pBAD_700 as described previously (8). Residues S189, G216, S217 and A226 play a critical role in determining the substrate specificity of the protease and were targeted for randomization (13). For protein expression, cells grown to an OD_{595} 0.5 were induced with 0.2% arabinose at 37 °C for 2 h. Cells expressing chylib1 were mixed with 1 % cells expressing WT chymotrypsin (ratio

determined by OD_{595}) and incubated with 20 nM Chy-BQ7 (a positively charged peptide substrate that contains a chymotrypsin-sensitive linker sandwiched between a FRET pair) for 15 min at 25 °C (8). The labeled population was analyzed and sorted using BD Jazz cell sorter (BD Biosciences, CA) at an event rate of ~7000/second, to isolate cells showing the highest (top 1%) fluorescence in the 530/40 nm channel (Figure 1A). In the absence of spiking, the frequency of cells expressing chylib1 in the sort gate was 0.3%. One thousand cells in the sort gate were collected in 100 μl of 2xYT media and plated onto LB-Cm plates. The resulting number of colony forming units (cfu) was less than 50. Poor viability of sorted cells (4 ± 1%) is possibly due to both proteolytic activity of chymotrypsin and labeling with peptide substrate in 1% sucrose.

To assess the effectiveness of our plasmid recovery method for sorted cells with poor viability, 50,000 cells in the sort gate were collected in a microcentrifuge tube and plasmid DNA was isolated using the buffer 3 protocol. Subsequent to transformation into *E. coli* MC1061 cells, the recovery efficiency (N_1/N_0) was estimated (by plating) at 63 ± 20%. Next, transformants recovered with 1 mL of SOC media (plasmid recovery method) or 50,000 sorted cells collected in 250 μl of 2xYT (culturing method) were directly grown in 100 mL of 2xYT media supplemented with 0.5% glucose and 25 μg/mL chloramphenicol at 37 °C for 10 h, and used to seed a subculture. After 2 hours of induction with 0.2% arabinose, cells were labeled with Chy-BQ7 and analyzed by flow cytometry. The presence of full-length chymotrypsin variants on the bacterial surface was characterized by incubating induced cells with 40 nM anti-FLAG - Phycoerythrin (ProZyme Inc., CA) for 30 min at 25 °C. The frequency of subpopulation in the sort gate increased by 35-fold in comparison to the parent population when plasmid recovery was employed (Figure 1A). On the other hand, the culturing method lost a majority of the cells expressing full-length chymotrypsin variants after just one round of sorting (Figure 1B).

We next performed similar experiments with our AraC-based biosensor system, in which cells expressing variant “AraC-TAL” activate GFPuv expression upon sensing triacetic acid lactone (TAL), while cells expressing wild-type AraC do not. *E. coli* HF19 cells harboring pPCC442 (P_{BAD}-*gfpuv*, RSF1030 origin) and pPCC423 (P_{tac}-*araC*, pBR322ΔROP origin, 30–60 copies/cell) or pPCC1202 (P_{tac}-*araC-TAL*, pBR322ΔROP origin) were induced with 5 mM TAL for 6 h at 37 °C (9, 14–16). Cells expressing AraC were spiked with cells expressing AraC-TAL (final composition of AraC-TAL = 10% as determined by OD_{595}) and sorted for cells showing the highest (top 2.5%) GFPuv fluorescence (Figure 2A). Sorted cells (N_0 = 500,000) were collected in 500 μL of SOC, and the resulting 2 mL of collected sample was centrifuged for 10 min at 17,900 xg. Decanting the supernatant left behind a residual volume (~200 μL) of concentrated cells for DNA isolation using the plasmid recovery method. Eluted plasmid was transformed into HF19 cells harboring pPCC442 with a recovery efficiency of 106 ± 52%. An aliquot of sorted cells was also directly plated to estimate viability. It is to be noted that the AraC system (6 ± 4%) had similar viability (based on cfu) to the chymotrypsin system (4 ± 1%). Transformants (plasmid recovery method) or sorted cells (culturing method) were directly grown in LB-Cm media supplemented with 50 μg/mL apramycin for 10 h at 37 °C and used for inoculating fresh 10 mL LB-Cm culture supplemented with 50 μg/mL aparamycin, 100 μM IPTG, 50 mM TES

and 1% glycerol to OD_{595} 0.2. Cells were induced with 5 mM TAL for 6 h at 37 °C (OD_{595} 10) and washed once with an equal volume of phosphate buffered saline for flow cytometry.

Using the geometric mean (GM) of clonal AraC-TAL fluorescence response to 5 mM TAL as the reference, a significant reduction ($p<0.05$) in GFPuv fluorescence signal (46 ± 18% of clonal AraC-TAL GM) was observed with the culturing method in comparison to the plasmid recovery method (89 ± 18%) (Figure 2A). Surprisingly, the culture method led to 43% of the population being unresponsive to both 5 mM TAL and 100 μM L-arabinose. Upon analysis of forward scatter – trigger pulse width plots, we observed that a majority of the sorted cells appeared 'larger' (Figure 2B). While the size of the cell can be linked to its growth rate in single cell organisms like *E. coli*, cell size also plays a fundamental role in fitness both directly and indirectly (17, 18). Since FACS based screening aims to select for the cells with the highest fluorescence, it is important to recognize that given the complex relationships between cell size, fitness and resistance, gene expression and plasticity (19, 20), it is possible to select for cells displaying high fluorescence that are not directly related to genetic variations in the protein of interest but rather due to variation in the host cell itself. While a more detailed mechanistic investigation needs to be undertaken to determine the relationships between high fluorescence, cell size and biphasic response to ligands as demonstrated in our model system, we note that the plasmid recovery method, because of retransformation into fresh cells after every sort, minimizes any advantages conferred by the host cell during culturing that are unrelated to genetic variations in the protein of interest.

In order to quantify the genetic diversity of gene variants obtained by plasmid recovery, we have undertaken high-throughput sequencing analysis of an AraC libraries being screened for responsiveness to TAL. In comparison to recovery of sorted cells by culturing, the number of unique AraC variants identified after sorting any given library using the plasmid recovery method was 5–6 fold higher (manuscript under preparation).

In summary, we have developed a technique for the direct isolation of medium-to-high copy-number plasmid DNA from FACS-sorted cells, with recovery efficiency sufficiently high for comprehensive screening of large, diverse libraries. This simple method for phenotype enrichment is well-suited as an alternative to culturing sorted cells or genotype recovery by PCR, especially when protein expression compromises host cell growth or viability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Method summary

E. coli cells were sorted using flow cytometry, and plasmid DNA directly isolated from sorted cells (10^5–10^6) was subsequently retransformed into competent cells. Our robust method yields high phenotype enrichment and provides an alternative to culturing sorted cells, when screening or target protein expression compromises host cell viability.

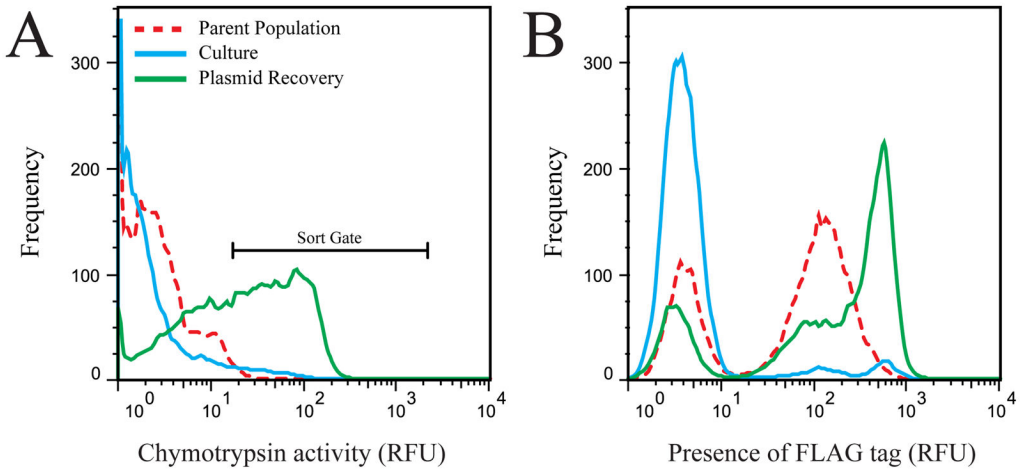

Figure 1.

Comparison of phenotype enrichment by plasmid recovery and culturing methods with chymotrypsin system. *E. coli* MC1061 cells expressing chylib1 were spiked with 1% cells expressing wild-type chymotrypsin and sorted to collect top 1% of the parent population (sort gate marked). Chymotrypsin activity (A) and presence of FLAG tag at C-terminal of full-length chymotrypsin (B) of parent population prior to sorting (red, dashed) and sorted populations recovered using plasmid recovery (green) and culturing (blue) methods measured using flow cytometry are shown.

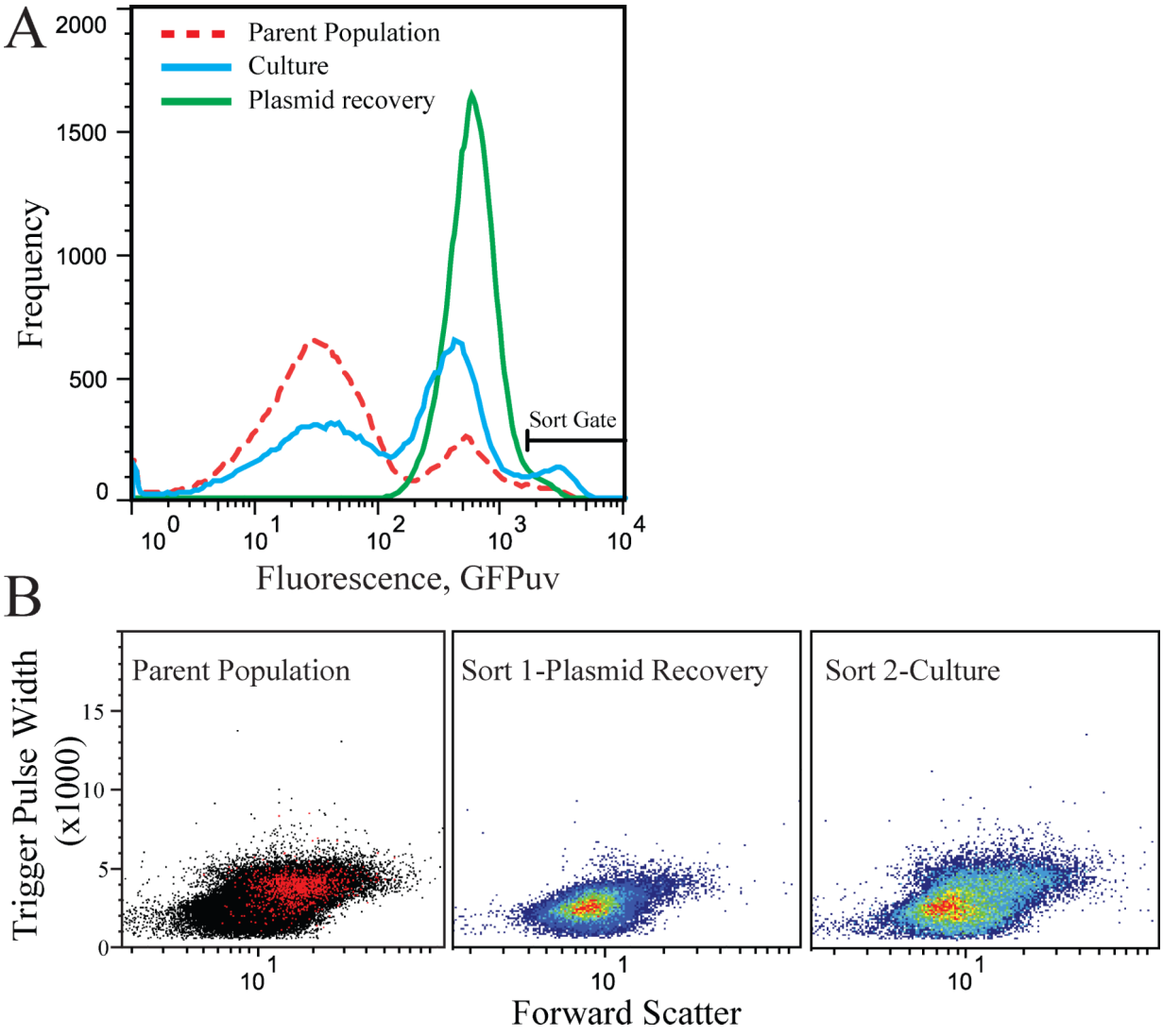

Figure 2.

Comparison of phenotype retained by plasmid recovery and culturing methods with the AraC biosensor system. (A) *E. coli* HF19 cells expressing wild-type AraC from plasmid pPCC423 were spiked 10% cells expressing AraC-TAL after induction with 5 mM triacetic acid lactone (TAL) and sorted to collect the top 2.5% of the parent population (sort gate marked). Histograms represent the frequency of cells showing fluorescence as a response to 5 mM TAL prior to sorting (red, dashed) and sorted populations recovered using plasmid recovery (green) and culturing (blue) methods. (B) Scatter plots representing the size of measured events, as determined by forward scatter and trigger pulse width. Plots show all events of the parent population (black) and the sorted subset in the sort gate (red). Representative density plots of populations recovered by plasmid recovery and culturing methods show frequencies of size distribution of populations.