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Rapid Peptide Reagent Isolation in a Disposable Microfluidic Cartridge

by Dimitra N. Stratis-Cullum, Joshua M. Kogot, and Paul M. Pellegrino

ARL-TR-5357 **September 2010**

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Sensors and Electron Devices Directorate, ARL

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14. ABSTRACT

In this collaborative development project, the challenges in affinity reagent isolation were addressed by combining two seemingly disjoint technologies—biosynthetic display libraries and microfluidic systems. To isolate optimal ligands from these libraries, our project developed an automated magnetic bacterial cell sorting system, termed micromagnetic cell sorter (MMS), to screen microbial libraries with unprecedented throughput using a disposable microfluidic cartridge. We report on the sorting system performance in rare cell and ultra-rare cell recovery (populations less than 0.001%), and compare the results to the standard manual method, magnetic activated cell Sorting (MACS). To demonstrate the system effectiveness in reagent isolation through sorting against an anthrax toxin target, protective antigen was performed and comparable results to standard screening methods were obtained. In addition, the good recovery performance through MMS selection yielded a consensus sequence among 24 unique binders.

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Reagents, peptide, affinity, microfluidics, protective antigen, cell sorting, bioassay, m olecular recognition elements, ICB

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Foreward

The U.S. Army Research Laboratory (ARL) was the government partner on a three-year, 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). This ICB 6.2 program enables transition of ICB 6.1 technologies, and in this project two 6.1 technologies (peptide display technology, and microfluidic cell sorting) produced an affinity reagent isolation platform. The purpose of this report is to highlight the Army evaluation of the affinity sorter technology produced during this program, and to provide recommendations for future work related to this area.

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1. Introduction

There are two main challenges in creating a high speed affinity ligand isolation technology against unknown/un-catalogued pathogens: (1) creation of a large (high diversity) and robust cell based library, and (2) creation of an ultrahigh throughput, disposable screening system. The approach developed in this work aims to address these challenges by combining two innovative technologies whose roots originate to the Institute for Collaborative Biotechnology's (ICB) 6.1 research program. In this report, we report on the sorting system performance in rare cell and ultra-rare cell recovery, and demonstrate the system effectiveness in reagent isolation through sorting against an anthrax toxin target, protective antigen.

Methodologies to develop highly specific affinity reagents for diagnostics, sensing, and therapy are critical to the U.S. Army mission in both the medical and nonmedical applications. With the development of hybridoma monoclonal antibody technology in 1975 by César Milstein and Georges Köhler (*1*), affinity reagents quickly became a cornerstone of diagnostic and sensing technology. Subsequently, a powerful new approach to more quickly generate affinity reagents, termed (bacterio) phage display technology, was developed by George Smith in 1985 (*2*). Phage display technology further transformed biotechnology by allowing researchers to generate tailor-made affinity reagents substantially faster than hybridoma technology and in as little as two weeks (figure 1).

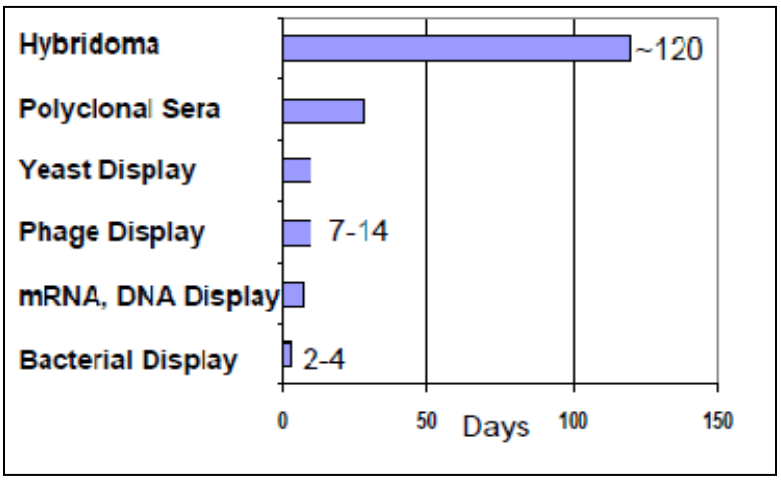

Figure 1. Estimated average time required for completion of ligand/affinity reagent development using existing approaches. Phage display assumes 3–5 rounds of selection by panning.

Despite recent advances in molecular recognition element alternatives, technology transfer to the Army has been challenging, and the current state-of-the-art continues to be limited by the high cost, limited availability, and often inadequate biochemical properties of affinity reagents for

many different applications. Given the enormous number of pathogens and toxins of concern to the Army, and their constant evolution, the demand for new reagents far exceeds the rate at which they can be produced. Consequently, methodologies to identify and produce ligands with high affinity and specificity to particular target molecules quickly, efficiently, reproducibly, and inexpensively are urgently needed.

Bacterial display technology offers an alternate strategy for generating tailor-made affinity ligands in a short time period (*3–4*). In this method, cellular machinery is used to generate billions of diverse polypeptide molecules that can be screened with high throughput methods to identify unique polypeptide sequences for a desired target (*4*). Currently, a number of systems, including messenger ribonucleic acid (mRNA) and ribosome display (*5*), eukaryotic virus display (*6–7*), and bacterial and yeast surface display (*3, 8*), are used to rapidly generate affinity reagents that can be used for diagnostics, proteomics, and therapeutic applications (*9–10*). Bacterial cell surface display is advantageous because the use of bacterial cells simplifies the polypeptide selection method and enables fast screening of potential recognition elements using fluorescence activated cell-sorting (FACS). Furthermore, polypeptide affinity reagents could offer more stable alternatives to antibody technology, enabling more rugged application in the field (e.g., enhanced thermal stability).

The development of a bacterial display system suitable for robust reagent discovery has proven challenging (*11*). Several different bacterial display systems have been reported (*12–14*); the broader utility of bacterial display, however, has been limited by technical problems, including accessibility on the cell surface and adverse effects on cell growth and viability (*11, 15*). Several groups have demonstrated that peptide libraries can be constructed in E. coli (*11*) as insertions in extracellular proteins such as pili or flagella subunits (*13*), or as insertions into outer membrane proteins (*16*). Though a few of these systems have provided encouraging results in a few different applications (*16, 17*), none of these bacterial display systems has been demonstrated to be suitable for routine isolation of high affinity peptide ligands that bind to arbitrary targets. Moreover, the construction of a large, high-complexity peptide library in a format that could serve as a single resource for many ligand isolation applications has not been reported.

Through the 6.1 research component of the ICB, the Daugherty Lab at the University of California-Santa Barbara (UCSB) achieved several technical breakthroughs in bacterial display, yielding a more robust peptide library methodology. Briefly, this peptide library employs an e-coli bacterial display platform, generated from the extracellular loop of OmpX outer-membrane protein (see figure 2). The randomized portion of the library is a15-mer, yielding a greater than 10^{10} member library.

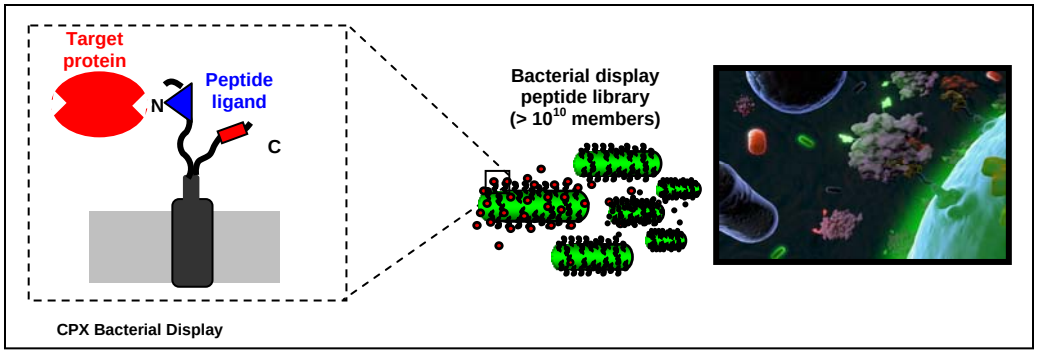

Figure 2. Schematic diagram of CPX bacterial library scaffold.

The design, construction, and application of the Daugherty display scaffolds have been well documented in a number of reports. (*4, 18–19*) In the work reported herein, this novel peptide library was used and combined with high throughput, on-chip cell sorter technology. It is important to note, however, that the automated micromagnetic cell sorter (MMS) platform can be adapted to other library technology (which was out of the scope of this work).

In table 1, a series of features of an ideal cell-sorting platform for microbial library screening is shown. Ideally, a system which is designed to sort quickly sort through a 10^{10} member library of potential binders must have both high-throughput and high purity, in addition to recovery sorting. The simplicity of operation is critical to practical application, and the integration of functions could ultimately allow for widespread, automated use. Finally, the ability to have a disposable interface would be advantageous to laboratories working with hazardous target material(s), to minimize human exposure and contamination.

Table 1. Desired features and importance in ligand isolation.

Desired Features	**Importance in Ligand Isolation**
High-throughput	Process libraries of 10^9–10^{11} cells in minimal time
High recovery	High statistical certainty of collection/low loss probability
High purity sorting	Collect target cells without non-target cell contamination
Simple, unattended operation	Allow for technology transfer/dissemination
Automation & scale-up	Generate ligands for many different targets
Integration of functions	Integrate clone isolation, sequencing, and characterization
Reusable or disposable	Process clinical or pathogen containing samples with minimal risk of cross-contamination

To isolate the bacterial clones that express peptide sequences with high affinity to the target, the conventional approach in this field uses multiple rounds of magnetic separation for pre-enrichment, followed by FACS sorting (*20–23*). Although this hybrid approach has proven to be effective manual magnetic sorting, is labor-intensive, and the sorting results are known to be

operator-dependent (*24*). Moreover, the high capital and maintenance cost of FACS instruments limit its accessibility. In addition, when dealing with infectious pathogens, a biohazard aerosol is potentially generated at the nozzle; additional steps need to be taken to reduce this hazard, such as adding an aerosol management unit, further increasing cost.

To address the need for a rapid, safe, efficient, cost effective, and reproducible method for peptide affinity ligand selection, our program developed an automated magnetic bacterial cell-sorting system, the MMS, equipped with disposable microfluidic cartridges shown in figure 3.

During the affinity ligand screening process (illustrated in figure 3), the target molecule is biotinylated and incubated with the bacterial display library. Cells displaying peptides that bind to the target molecule become labeled with biotinylated target. The resulting target-binding cells are incubated with streptavidin-coated magnetic beads and captured by the magnetic particles through biotin-streptavidin binding (*4*). After sample preparation, MMS sorting is performed to isolate the magnetically-labeled bacterial clones from the rest of the library. The collected cells were cultured overnight for processing in the next round of selection. The low cost disposable cartridge developed herein aims to mitigate hazard exposure through controlled containment of the hazard through selection and disposal, with minimal handling by laboratory personnel.

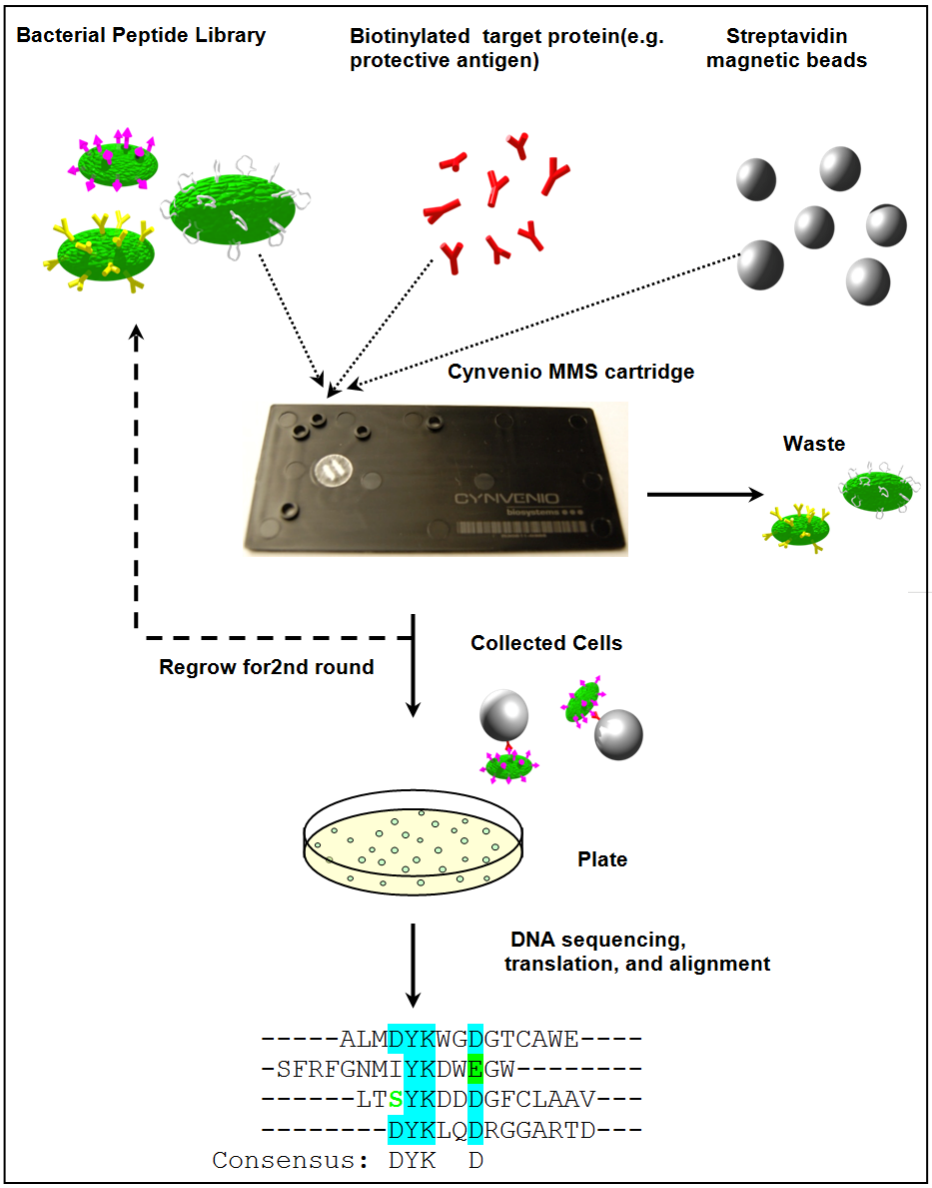

Figure 3. Schematic description of peptide library screening using Cynvenio's micromagnetic sorter (not to scale).

The target protein (e.g., protective antigen) is biotinylated and labeled with streptavidin-coated superparamagnetic beads; thus, bacterial cells displaying peptides that have affinity to target protein are captured on the superparamagnetic beads, allowing continuous-flow separation by Cynvenio MMS. The design also allows non-binding bacterial cells flow out as waste. The binding population is eluted from the MMS cartridge, and can either be amplified by overnight culture growth for a further round of labeling and sorting, or plated on solid media to isolate single clones for sequence determination. An example of the typical results of DNA sequencing, translation, sequence alignment, and consensus determination is shown for clarity.

Experiments were conducted on the MMS system to determine the rare cell and ultra-rare cell recovery (populations less than 0.001%) (*25–26*) capability of the instrument compared to manual MACS. Rare-cell isolation has potential in medicine for cell identification, such as cancer cell isolation and population enrichment (*27–29*), and could have potential military and national security applications for isolation of potential pathogens.

As a further demonstration of the MMS system's effectiveness for binder isolation, protective antigen (PA) of an anthrax toxin (*Bacillus anthracis*) was chosen as a target. The CytomX Therapeutics eCPX bacterial display library, expressing ~3 x 10^{10} discreet random peptides, was screened for affinity reagents, which bound to protective antigen.

2. Experimental

2.1 Micromagnetic Cell Sorter (MMS) System

The MMS is an automated magnetic separation system consisting of a disposable microfluidic cartridge (figure 4a) and a companion instrument (figure 4b). The disposable cartridges are made of injection-molded polypropylene (Pinnacle Polymers PP 5135C). The 200-µm deep fluidic channels are defined by two injected parts, which are laser-welded (California Lasers, Simi Valley, CA), and a portion is heat-staked with a hydrophobic membrane for bubble removal (Pall Co, Ann Arbor, MI). The trapping region was designed to accommodate up to 1×10^9 of 1 µm trapped magnetic beads and process up to 1×10^{11} bacterial cells. Female luer fittings on the top of the cartridge allow for a leak-proof interface between the cartridge and disposable syringes (Becton Dickinson, San José, CA). The luer fittings on the cartridge are designed to hold a reservoir array for pneumatically driven applications, as well as the injector inputs. There are a total of four luer ports required for two sample injectors (1 or 5 mL volume), one running/wash buffer injector (up to 10 mL) and one elution buffer injector (up to 3 mL volume). Strategically designed micro-channels allow for full automation of magnetic separation on the cartridge. To accomplish this, five pneumatically actuated pinch valves are located on the underside of the cartridge, allowing for the redirection of flow. These valve membranes require a force of ~15 lb/in^2 to seal and are robust enough to be actuated multiple times.

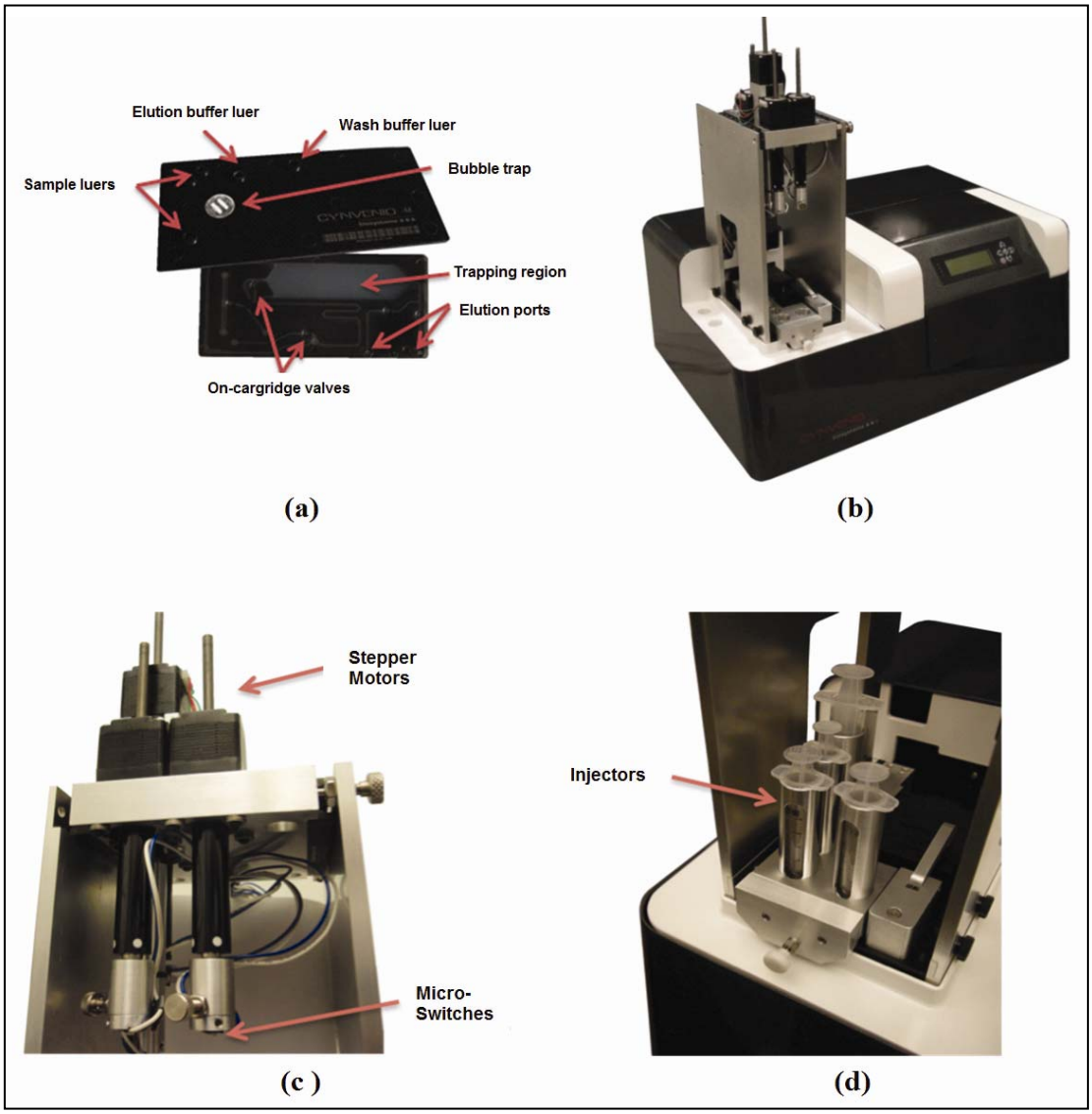

Figure 4. Photos of the micromagnetic sorting (MMS) platform. (a) MMS disposable cartridge with front side showing sample luer interfaces and backside showing separation region, on-cartridge valves and fluid path ways; (b) automated MMS instrument with an volumetric control module to precise fluid injection speeds and volumes, so that the separation results were highly repeatable independent of sample and buffer viscosity, or manufacturing variations in the disposable cartridge; (c) Stepper motors are implemented to actuate injectors with micro-switches for injector location sensing; (d) Off-shelf syringes are used as injectors for volumetric sample injection.

The instrument uses a cRIO controller with LabVIEW script (National Instruments, Austin, TX), outfitted with standard digital and analog in/out modules for control of the internal components. Flow rates within the cartridge are controlled by four stepper motors (figure 4c) and controller boards (Haydon and Anaheim Automation, respectively), which physically push on the injectors (figure 4d). These motors are fitted with micro-switches (Panasonic ECG, Secaucus, NJ) that allow for the automatic calculation of input volume. Valves on the cartridge are actuated using pneumatically controlled air cylinders (SMC Corp, Noblesville, IN) and a DC diaphragm pump

(Thomas provided by Nor Cal Controls, San José, CA). There are 70 custom neodymium-iron boron magnets, which are position-controlled by another Haydon stepper motor. The magnets are distributed equally among top and bottom portions of a magnetic rack, which sandwich the disposable cartridge. A single motor, in conjunction with a spring, allows for both horizontal and vertical movement of magnets. This facilitates horizontal movement required for trapping and elution, and vertical movement capable of agitating the sample within the cartridge. Software control is provided using a LabVIEW interface. Push-button applications have been created for bacterial library sorting. Advanced users can generate custom sorting routines, which allow full access to all the operation parameters with minimal training. Direct control of flow rates, wash stringencies, and positive/negative selection criteria enables the end-user to specify the magnetic bead and protocol of choice, and optimize it for applications beyond bacterial library sorting (i.e., cell culture, flow cytometry, toxicology studies, etc.).

2.2 Sorting Procedures and Sample Preparation

Display Library

A bacterial display library (Cytomx Therapeutics eCPX library) that contained approximately 3 $\times 10^{10}$ members was screened for clones that display PA binding peptides The random library is first grown in 500 mL LB media containing 25 µg/mL chloramphenicol (LB-Cm^{25}) to an OD_{600nm} of approximately 0.6 (Eppendorf Biophotometer; Eppendorf, Hamburg, Germany). At this point in exponential growth phase, the cells were induced by the addition of arabinose to a final concentration of 0.04% (w/v); the enhanced circularly permuted OmpX (eCPX) gene expressing the library peptides was under the control of an arabinose inducible promoter (*30*). The cells were shaken at 37 °C for an additional 45 min, after which the OD_{600nm} was again measured, and, using the assumption that an OD_{600nm} of 1.0 relates to a bacterial concentration of 1 x 10^9 cfu/mL, approximately 2 x 10^{11} cells were pelleted by centrifugation at 3000 g for 20 min.

SA-binder depletion

The bacterial pellet was re-suspended in 1.5mL of PBSB (PBS buffer plus 0.5% BSA) containing 1 × 10^9 paramagnetic beads (Invitrogen DynabeadsMyOneStreptavidin C-1; Invitrogen, Carlsbad, CA). The cell suspension was incubated at 4 ºC for 45 min, with rotation to allow depletion of streptavidin binders from the library prior to selections. To remove these beads and any cells bound to them, the sample was loaded onto an MMS cartridge and separated at a sample flow rate of 50 mL/hr and buffer flow rate of 10 mL/hr. The MMS cartridge captured the unwanted bead bound cells and allowed collection of the depleted library ready for enrichment. For the SA binder depletion using a benchtop magnetic bead separator (manual MACS), the bacterial cell pellet with 1 × 10^9 paramagnetic beads was pelleted using a magnet next to the tube. The magnetic separation was performed for 5 min to allow the bead pellet to form. The sample was washed and aspirated with 5 × 1 mL PBS washes, and resuspended in 1 mL PBSB for PA binder enrichment.

PA-binder enrichment

The SA-binder depleted library was centrifuged at 3000 g for 20 min, resuspended in 1 mL PBSB buffer containing 600 nM biotinylated protective antigen (List Biological Laboratories, Inc; Campbell, CA), and incubated at 4 ºC for 45 min. Cells were centrifuged as previously described and re-suspended in 1 mL PBSB buffer with 1×10^9 pre-washed magnetic beads. After 45 min at 4 ºC with rotation, the cell-beads suspension was loaded into an MMS cartridge (or separated by manual MACS using the same methods as SA binder depletion). Bacterial cells bound to PA were trapped on cartridge and then eluted into a collection vessel. A second round of sorting was performed, following the same protocol as the first; however, the assay parameters were adjusted to account for the smaller starting population and to increase the selection pressure in the second round. Therefore, we used 1×10^8 cells in 50 µL of 300 nM PA and 1×10^8 magnetic beads. Cells were incubated static on ice for all labeling steps. Also, 1 µM biotin was added in the washing buffer to compete with any remaining streptavidin binders (peptides which bind to streptavidin typically have a much lower affinity than biotin). In the third round of MMS sorting, cells were labeled with 150 nM biotinylated PA, and then labeled with 1×10^6 magnetic beads in 50 µL of PBSB. After each round of magnetic separation, the bead-bound enriched library was added to LB-Cm^{25} media, supplemented with 0.2% glucose to inhibit expression of the eCPX gene and, therefore, prevent growth bias. The cultures were then grown overnight at 37 ^{o}C with shaking.

Analysis of PA binder enrichment by FACS

To quantify the library enrichment of potential PA binders, we performed FACS analysis (BD FACSAria; BD Biosciences, Franklin Lakes, NJ) using biotinylated PA (EZ-Link Sulfo-NHS biotinylation kit; Thermo Scientific, Rockford, IL) labeled with alternating fluorescent secondary labels—streptavidin, R-phycoerythrin conjugate (SAPE; Invitrogen, Carlsbad, CA), anti-biotin-phycoerythrin (Miltenyi Biotec; Bergisch Gladbach, Germany), and Neutravidin, R-phycoerythrin conjugate (NAPE; Invitrogen, Carlsbad, CA)—similar to previously published procedures (*4*, *12*). Following each round of PA selection, the arabinose-induced cell population was incubated with 100 nM biotin-PA solution for 45 min. The sample was centrifuged at 3000 g for 10 min to remove unbound biotin-PA, and was resuspended in a 25 µL solution of PBSB with secondary label concentration of 5 µg/mL and incubated for 45 min at 4 ºC. The sample was centrifuged and resuspended in 1 mL ice-cold BD FACSFlow (BD Biosciences, Franlin Lakes, NJ) sheath immediately prior to FACS analysis. Cells labeled with SAPE exhibit increased red fluorescence and are easily distinguishable by flow cytometry.

Ultra-Rare Cell Recovery

To measure the rare and ultra-rare cell recovery of the MMS, cells not expressing surface display peptides (negative control) were doped with a known quantity of cells expressing a known PA binding sequence. A 1×10^{-3} or 0.1% PA binder (1 µL PA binding bacteria in 1mL on negative control bacteria) sample was diluted in negative control library to create samples ranging from

1×10^{-3} to 1×10^{-8}, or 0.0000001% PA binding cells. The samples were analyzed by FACS before and after MMS (or MACS) sorting to determine the ultra-rare cell recovery capability of each technique.

3. Results and Discussion

Three key parameters are used to evaluate cell sorting which is required for binder isolation. The first is throughput, which measures how many cells can be sorted per second. As discussed previously, the MMS platform is designed for high throughput screening since it is capable of screening a bacterial library containing 3×10^{10} members in 15 min. The second is *purity*, (the fraction of collected cells which actually bind the target), and the third is *recovery* (the fraction of binders collected relative to the total number of binders in the naïve library).

To evaluate the purity of the isolated fractions, we performed FACS analyses of PA binder populations in the positive sorting sample and the negative control sample. Figure 5 shows the FACS analysis results of the fraction of target-binding clones in the enriched population after incubation with fluorescently labeled PA protein target. The intensity of red fluorescence (x-axis) represents the level of binding on the cell surface, which may be due to either a high expression or a high affinity for the target. To assess recovery, serial dilutions of the collected bacterial populations were grown for 14 to 18 h on LB-Cm^{25} plates at 37 ºC. The serial dilutions enable a calculation of resultant library diversity compared to the original eCPX library diversity of (3×10^{10}) and provide verification that enrichment has occurred after each round of selection.

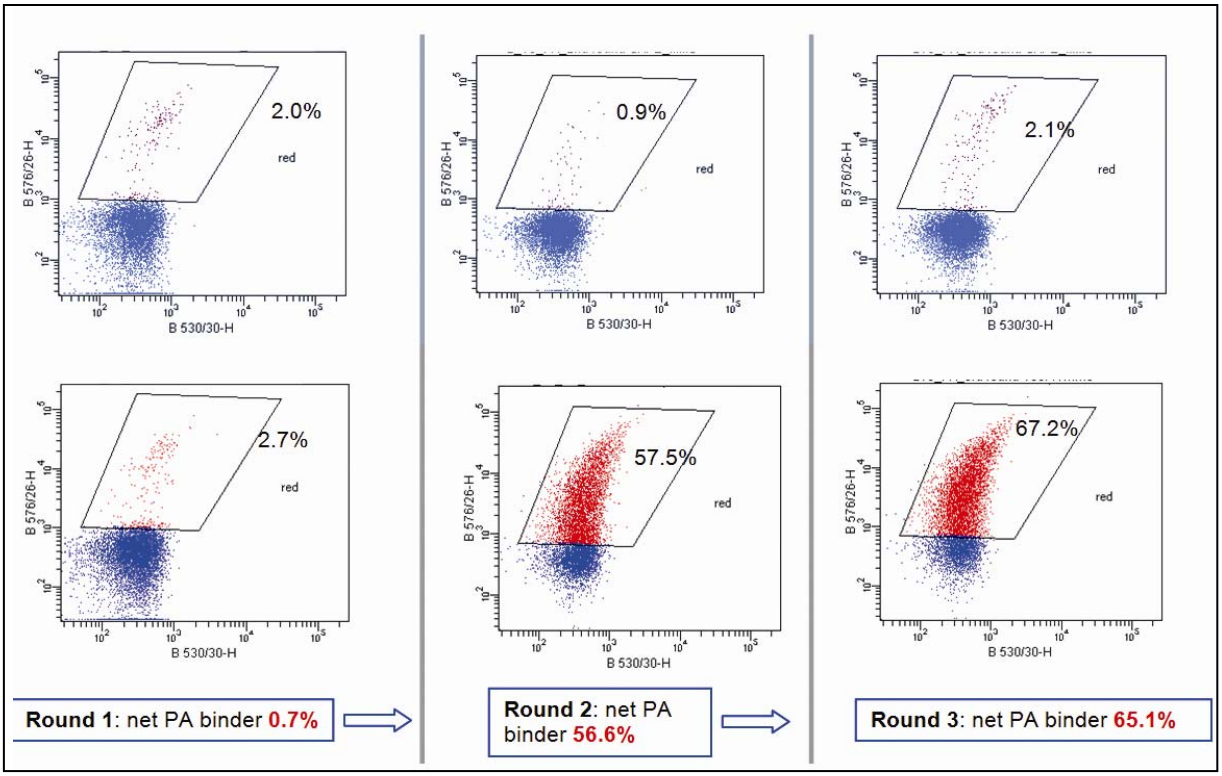

Figure 5. FACS analysis of the fraction of target-binding clones in the enriched population after incubation with fluorescently labeled PA protein target over the course of three rounds of selection. Following one round of MMS, 0.7% (net) of the population exhibit PA binding peptides. Following two rounds of MMS, 56.5% (net) of the population exhibit target-binding peptides. 65.1% (net) of the population exhibit target-binding peptides after three rounds MMS selection.

FACS analysis shows that after one round of MMS selection, the frequency of cells capable of binding to PA reached 0.7%; the second and third rounds further enriched the population to 56.1% and 65.5%, respectively, with MMS selection (figure 5). Individual clones were picked at random from the positive populations and sequenced (Genewiz, South Plainfield, NJ). Sequences were analyzed and aligned using the Vector NTI software suit (Invitrogen, Carlsbad, CA). Sequencing of 24 clones obtained after three rounds of sorting yielded 15 clones displaying a consensus motif **WXCFTC**. After repeating the selection process, a total of 24 distinct peptide sequences showed the **WXCFTC** consensus (figure 6). This consensus was the same as the best binder found with the conventional MACS/FACS approach SM545 (GSFYDSIL**FY**C**MTC**R). Ten randomly selected clones, expressing peptides with the consensus sequences, all show binding to 150 nM PA as measured by flow cytometry; results for three of the clones are shown in figure 7.

```
    yz100  FGLDSMQTSWYCFTC---------
    yz140  ------LSSWICFTCEVETVK---
    yz106  ---------FFCFTCHSPPLLALS
    yz121  -----RQLAYICFTCNTTNV----
     yz59  ---VFYPAAYFCFTCNND------
     yz97  ---------FFCFTCVSTQWSSLI
    yz127  ----SLLTAFSCFTCGVSL-----
    yz139  ---------FSCFTCFIDWSGGSS
     yz88  ------DVSFSCFTCAPYAFA---
    yz107  ------VPLYYCFTCSGYPSS---
     yz62  ---IWANSTYYCFTCNLQ------
    yz142  --VPAVRSTWYCFTCSL-------
     yz72  -------SPWICFTCPLIKLTS--
    yz105  --------NWLCFTCGMDFIDPD-
    yz133  ---TQFYEEWYCFTCHAD------
    yz109  ---NVEHISWSCMTCLYS------
    yz128  ------PISWLCFTCLIHSVG---
    yz111  ---------WVCYTCSFRVPNPIQ
    yz118  ---NYAYVHWLCWTCTDR------
    yz116  ---SGQCFQWGCYLYAPP------
    yz141  ------YESWFCYTCIKEEVM---
     yz64  ---------WFCYTCWAEVLPHPS
     yz91  --------NWTCFTCVYRPGNYV-
     yz99  --WIYVPTQWTCFTCSQ-------
Consensus           W CFTC
```

Figure 6. Peptide sequences of clones selected by MMS system for binding to protective antigen, revealing a six residual consensus sequence of WXCFTC among 24 individual clones.

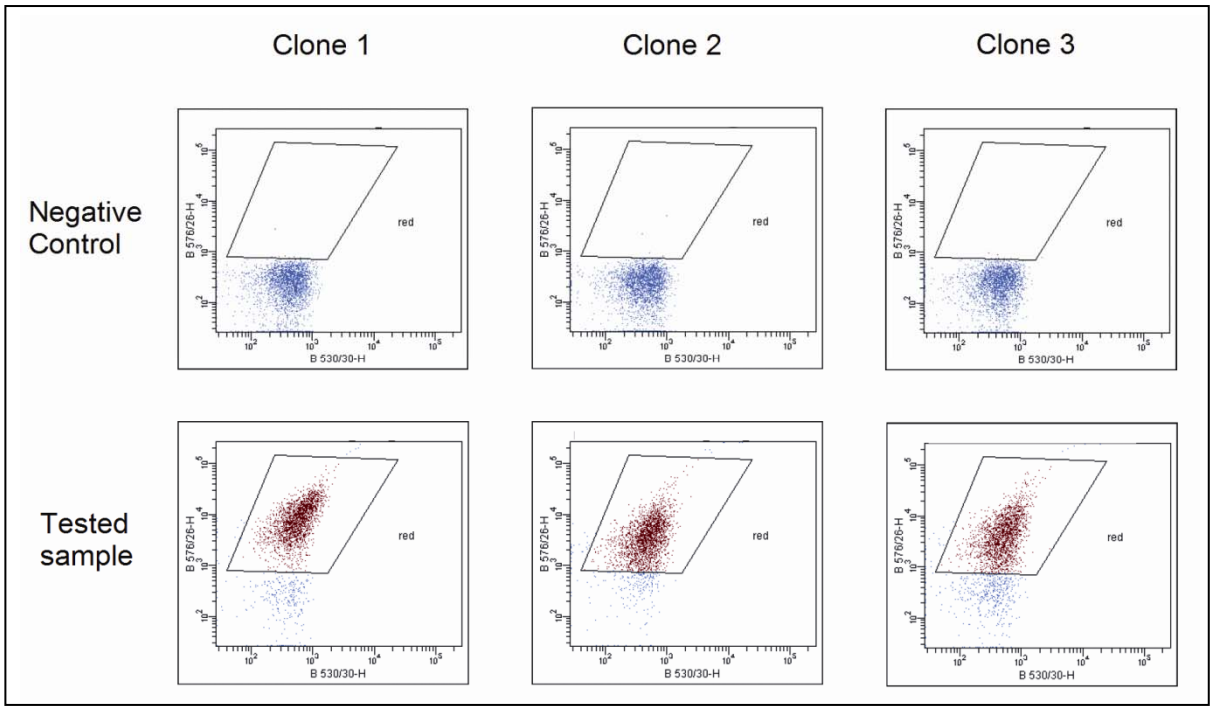

Figure 7. FACS analysis of three selected single clones labeled with biotinylated protective antigen. The negative control (top row image) was run with secondary label alone (SAPE); the test samples (bottom row image) were labeled with 150nM PA followed by SAPE. All of the tested samples show binding to PA as evidence by the increased positive signal (increased population in gate).

Both MMS and MACS show the capability of ultra-rare cell recovery of 3.00% for the MMS and 5.68% for the MACS at 1 x 10^{-8} cells (table 2), which represents a greater rare cell population than any of the presort populations measured. The MMS is more consistent than the MACS at recovering all ultra-rare and rare cell populations, given the lower standard deviation for recovery for nearly all the samples tested (except at 1 x 10^{-7}, which has a lower standard deviation with the MACS). Overall, the MMS proved to have less variance between rare cell recovery experiments than the MACS, given that the average standard deviation for the MMS was only 8.6%, compared to 21.8% for MACS for all rare cell populations between 0.1% and 0.0000001%.

Table 2. Rare-cell recovery results as % recovered for the comparison of the MMS and manual magnetic sorter (MACS). Although both MMS and MACS show the capability of rare-cell recovery to 10^{-8} cells, the most reliable level for rare-cell recovery in a single magnet sort according to the large variance is 10^{-6} for the MMS and 10^{-5} for the MACS, as determined by the increased variance next cell recovery sample. The average standard deviation across all samples in the 4 independent trials for the MMS is 8.6% compared to 21.8% for the MACS.

	Trial 1		Trial 2		Trial 3		Trial 4		Average ± SD		
	MMS	MACS	MMS	MACS	MMS	MACS	MMS	MACS	MMS	MACS	Presort
10^{-3} (0.1%)	96.2	0	91.1	11.9	96.9	97.7	97.7	96.9	95.48 ± 2.98	68.83 ± 52.97	2.83 ± 1.67
10^{-4} (0.01%)	95.6	94.9	69.2	42.2	90.9	82.3	87.7	76.3	85.85 ± 11.56	73.93 ± 22.53	1.30 ± 0.44
10^{-5} (0.001%)	26.2	82.9	39.9	5.2	28.2	25.1	67.6	40.7	40.48 ± 19.07	38.48 ± 32.99	0.97 ± 0.29
10^{-6} (0.00001%)	18.2	34.0	15.1	4.8	3.6	9.2	0	4.3	9.23 ± 8.79	13.08 ± 14.12	1.03 ± 0.46
10^{-7} (0.000001%)	2.1	1.6	13.7	5.6	0	8.1	1.1	6.5	4.23 ± 6.37	5.45 ± 2.77	1.17 ± 0.49
10^{-8} (0.0000001%)	1.3	1.0	6.9	8.2	3.8	8.6	0	4.9	3.00 ± 3.04	5.68 ± 3.53	1.10 ± 0.71

4. Conclusion

The U.S. Army Research Laboratory (ARL) was the government partner on a three-year, 6.2 Research Project funded through the Army university-affiliated research center (UARC), the ICB at UCSB under grant DAAD19-03-D-0004). During this program, we transitioned two 6.1 technologies (peptide display technology, and microfluidic cell sorting) and produced an affinity reagent isolation platform. In this report, we highlight the first phase of the Army evaluation of the affinity sorter technology.

Characterization of the MMS system's performance was achieved by screening against PA from *Bacillus anthracis*. Automated MACS are not currently available for display library screening. Therefore, results obtained using the MMS platform were directly compared to those obtained using conventional MACS/FACS sorting. In this work, we demonstrated that the automated MMS platform is capable of effectively enriching affinity peptides against potential biological warfare agents with high throughput. For a typical 1 mL sample volume, MMS requires only 5 min of user interaction, while manual selection requires more than 20 min.

With regard to gross throughput per hour, MMS is able to process 5×10^{12} cells/hr (50 mL/hr at a cell concentration of 1×10^{11} cells/mL), which is four orders of magnitude higher than that achieved using state-of-art FACS instrumentation or a previously reported dielectrophoretic cell sorter (*31*). In addition, for the first time, a PA binder sequence consensus WXCFTC was discovered via the high recovery performance of MMS, which results from the MMS sorting system's automation and integrated nature. Furthermore, the sorting protocols described here can be easily adapted to select other affinity reagents to targets of interest.

The current results not only demonstrate the potential of the MMS platform for automated reagent discovery, but could lead to a much broader extension to a variety of applications requiring rare-cell recovery that were out of the scope of our objectives. For example, the ability to consistently recover and isolate a rare cell population from a large negative control population provides a useful method for pathogen detection in food and water using this low cost, disposable cartridge system. The use of a disposable cartridge permits the analysis of potentially hazardous materials with minimal user exposure, and eliminates any concerns for cross-contamination of samples. The MMS sorting platform is also suitable for general magnetic cell-sorting, with applications ranging from mammalian cell separation to protein and nucleic acid purification.

5. Future Work

Although the MMS sorter development shows promise for affinity reagent isolation applications, there are a number of areas of ongoing work, as well as recommendations for future research and development, that can be made. Our current and future work related to this program includes the continued evaluation of binder candidates selected against protective antigen (off scaffold) and other targets (on and off scaffold) in terms of binding affinity and specificity. The PA peptide binders produced during this work show promise, as the binding affinity on scaffold and in preliminary work in peptibody fusions show Kds in the nM range. They exhibited little to no cross-reactivity with IgG and other standard protein systems typically used in immunoassay chemistries, but extensive cross-reactivity studies are still underway. Also, we are currently evaluating Kds of the free, solubilized peptides off scaffold using standard enzyme-linked immunoassay methods (ELISA). We plan to follow up with another report detailing the peptide analysis of these PA binders.

We plan to not only extend the sorting to other target analyte materials, but also explore the use of alternate library technologies (e.g., yeast library display, aptamers, etc.) with the MMS isolation.

As discussed earlier, a main challenge in affinity reagent isolation from bacterial libraries is overcoming the tedious steps involved in manual selection that leads to irreproducible results.

Although advances are made herein towards automating this application, the final binder population selection is expected to be representative of the "family" of binders exhibiting desired characteristics. However, statistically, the best binders can be lost through adsorption, etc. Therefore, additional optimization of the binder population is still needed, such as employing traditional soft randomization techniques and re-selection.

Additional work related to the challenge of understanding molecular recognition includes a new, three-year program, beginning in FY10, funded by the Defense Threat Reduction Agency, (DTRA) termed "Iterative Modeling of Peptide-Protein Interaction for 'Smart' Reagent Development." We have chosen the SM545 PA peptide reported here as a model system and aim to ultimately develop a comprehensive modeling toolkit that can be used to predict the best (equivalently optimized) smart binders after initial selection.

Finally, the MMS was very simple to operate; however, the system could benefit greatly from further engineering to help with reproducible card placement and full automation. No current plans by the industrial partners exist to further develop the platform specifically for bacterial library screening, but advances in the fluidics and other cartridge capabilities are underway for other commercial applications.

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List of Symbols, Abbreviations, and Acronyms

ARL	U.S. Army Research Laboratory
DTRA	Defense Threat Reduction Agency
DNA	deoxyribonucleic acid
ECBC	Edgewood Chemical Biological Center
ELISA	enzyme linked immunoassay
FACS	fluorescence activated cell sorting
ICB	Institute for Collaborative Biotechnologies
IgG	immunoglobulin G
LB	lysogeny broth
LB-Cm^{25}	lysogeny broth with with 25 μg/mLchloramphenicol
MACS	magnetic activated cell sorting
MMS	micromagnetic cell sorter
MRE	molecular recognition element
mRNA	messenger ribonucleic acid
NAPE	neutravidin, R-phycoerythrin
PA	protective antigen
PBSB	phosphate buffered saline with 0.1% bovine serum
SAPE	streptavidin, R-phycoerythrin
UARC	university-affiliated research center
UCSB	University of California-Santa Barbara

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