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Automated Panning and Screening Procedure on Microplates for Antibody Generation from Phage Display Libraries

LAURA TURUNEN, KRISTIINA TAKKINEN, HANS SÖDERLUND, and TIMO PULLI

Antibody phage display technology is well established and widely used for selecting specific antibodies against desired targets. Using conventional manual methods, it is laborious to perform multiple selections with different antigens simultaneously. Furthermore, manual screening of the positive clones requires much effort. The authors describe optimized and automated procedures of these processes using a magnetic bead processor for the selection and a robotic station for the screening step. Both steps are performed in a 96-well microplate format. In addition, adopting the antibody phage display technology to automated platform polyethylene glycol precipitation of the enriched phage pool was unnecessary. For screening, an enzyme-linked immunosorbent assay protocol suitable for a robotic station was developed. This system was set up using human γ -globulin as a model antigen to select antibodies from a VTT naive human single-chain antibody (scFv) library. In total, 161 γ -globulin-selected clones were screened, and according to fingerprinting analysis, 9 of the 13 analyzed clones were different. The system was further tested using testosterone bovine serum albumin (BSA) and β -estradiol-BSA as antigens with the same library. In total, 1536 clones were screened from 4 rounds of selection with both antigens, and 29 different testosterone-BSA and 23 β -estradiol-BSA binding clones were found and verified by sequencing. This automated antibody phage display procedure increases the throughput of generating wide panels of target-binding antibody candidates and allows the selection and screening of antibodies against several different targets in parallel with high efficiency. (*Journal of Biomolecular Screening* 2009:282-293)

Key words: antibody phage display, automation, high throughput, magnetic beads, robotic station

INTRODUCTION

THERE IS A GROWING NEED FOR NEW SPECIFIC BINDERS to be used for molecular recognition in various fields of biotechnological and biomedical science. Antibodies are often the choice of scaffold for the binding molecules because they are well characterized, and in vitro methods for obtaining them have been developed extensively.¹ The use of antibody phage display has been recently reviewed by many authors, from the therapy² and especially from the proteomics point of view.^{1,3-5} The focus of method development has been shifted to more robust approaches to fulfill the growing needs of proteome-wide research.⁵ Isolation of antibodies from phage display libraries can be carried out in automated procedures, ensuring that new antibodies can be obtained within a reasonable time span.^{2,3,6,7}

Automation and several high-throughput methods have been applied to different steps of antibody phage display technology. The use of magnetic beads and an automated bead processor

increase the throughput and efficiency of the phage enrichment step.⁸ This approach has been used successfully in high-throughput screening (HTS) of phage display libraries.⁸⁻¹⁰ One approach to enable panning against several antigens in parallel has been referred to as the multipin method, using a 96 array of antigen-coated pins in selection.¹¹ Krebs et al.⁶ described automated procedures for panning and screening, in which the growing and selection of phages are performed in the microplate format. This allows the easy handling of multiple samples in parallel with multichannel pipetting devices. One high-throughput approach for panning with respect to the antigen is selection of phages on a filter of a 2D gel¹² or high-density Western blot.¹³ SPOT peptide arrays also can be used for phage selection.¹⁴ Hallborn and Carlsson⁷ pushed the throughput of screening so far that 10,000 clones can be screened automatically per day, and the downstream characterization of the selected antibodies becomes the rate-limiting step. The throughput of the screening can be further increased by using the array format.^{15,16} On arrays, enzyme-linked immunosorbent assays (ELISAs) can be performed in high density on filters¹⁷ or glass slides.¹⁸ The bottlenecks are in the supply of targets³ and in the production, purification, and detailed characterization of the antibodies.²

A recently emerged high-throughput technique applied to the screening and characterization of new ligand-binding molecules is a multiplexed bead-based assay format using flow

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cytometry.¹⁹⁻²¹ Using optically labeled sets of beads, it is possible to screen dozens of binding events determining specificity and/or cross-reactivity in a single tube and in minimal time. Another very recent high-throughput technique applies fluorometric microvolume assay technology (FMAT) to hybridoma screening against cell surface antigens²² or bead-bound toxin antigen.²³ The advantages of this system include reduced antigen consumption, homogeneous assay format, and decreased hands-on time performing the assay.

We combined panning with a magnetic bead processor and growing of the phages on 96-well plates to an automated screening process using a robotic system. The conventional polyethylene glycol (PEG) precipitation of phages between selection rounds was found to be unnecessary, which allowed the enrichment of the phage library with several antigens in parallel. An efficient protocol was developed for the robotic ELISA screening. As a result of optimization, we have a robust method for generating antibodies against several targets in parallel, requiring only one pair of hands. The system is very well suited for small laboratories producing antibodies against variable sets of antigens.

MATERIALS AND METHODS

Escherichia coli strains and media

Escherichia coli strain XL1-blue (Stratagene, La Jolla, CA) was used as a host for phage display and antibody expression. VCS-M13 (Stratagene) was used as helper phage. The cultivation medium was Superbroth (SB, 2% yeast extract, 3% tryptone, 1% MOPS, pH 7) supplemented with 50 µg/mL carbenicillin and 10 µg/mL tetracyclin. Agar plates were made in Luria Broth (LB, 0.5% yeast extract, 1% tryptone, 1% NaCl) supplemented with 100 µg/mL ampicillin (LB-amp).

Automated equipment

The magnetic bead processor used for automated panning was KingFisher (Thermo Scientific, Waltham, MA), capable of moving magnetic beads between 8 wells of 24 strips in parallel.

The robotic system consisted of an ORCA Robot Arm, Multimek 96 and Biomek 2000 pipetting stations, Lidding station (Beckman Coulter, Fullerton, CA), ELx405 Micro plate washer (BioTek, Winooski, VT), 2 Multidrop 384 dispensers (ThermoElectron, Waltham, MA), and a Wallac Victor2 V plate reader (PerkinElmer, Waltham, MA) controlled by SAMI software (BeckmanCoulter). All the equipment was operated at room temperature.

Phage antibody library

The naive human scFv antibody library used in this study was prepared earlier. It has been used to isolate antibodies against different antigens by manual panning.²⁴⁻²⁶ Briefly, the antibody

genes were cloned from a pool of human lymphocytes of 50 healthy donors. After mRNA isolation and cDNA synthesis, the repertoire containing the variable regions of heavy chains (V_H) was prepared by amplifying the IgM-specific cDNA. The light chain repertoires were obtained by amplifying both kappa (V_K) and lambda (V_λ) specific cDNAs separately. The heavy chains were combined with the light chains to create 2 libraries, $V_H V_K$ and $V_H V_\lambda$, both containing approximately 10^8 clones.

For preparation of scFv display libraries, 0.5 µg of each library DNA was electroporated into 100 µL of competent XL1-Blue cells with a transformation efficiency of 1.3×10^9 plaque-forming units (pfu)/µg pUC plasmid DNA. After transformation, 2 mL SOC (0.5% yeast extract, 2% tryptone, 1 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 0.4% glucose) was added, and the culture was shaken at 200 rpm for 1 h at 37 °C. The culture was diluted to 10 mL with SB containing 20 µg/mL carbenicillin and 10 µg/mL tetracyclin and shaken for 1 h. The carbenicillin concentration was increased to 50 µg/mL, and after 1 h of shaking, about 3×10^{11} pfu VSCM13 helper phages were added. The culture was incubated without shaking for 15 min at 37 °C and then diluted to 100 mL with SB containing the antibiotics as mentioned above. Shaking was continued for 2 h, after which kanamycin was added to 70 µg/mL, and cultivation was continued at 34 °C overnight.

Phages were recovered from the culture supernatant with PEG precipitation. The culture was centrifuged at 2600 g for 15 min at 4 °C. The supernatant was mixed well with 25 mL (1/4 volume) of cold PEG/NaCl solution (20% PEG 8000/2.5 M NaCl). After incubation on ice for 30 min, the suspension was centrifuged at 13,000 g for 20 min at 4 °C to pellet the precipitated phages. The supernatant was discarded and the phage pellet suspended in 2 mL phosphate-buffered saline (PBS; 15 mM sodium phosphate, 150 mM NaCl, pH 7.4). Remaining cell debris was removed by centrifugation in Eppendorf tubes at 20,000 g, and phages were precipitated again from 1 mL of supernatant by adding 250 µL PEG/NaCl, incubating on ice for 15 min, and centrifuging for 5 min at 20,000 g. The pellet was resuspended in 1 mL 0.5% bovine serum albumin (BSA)/PBS and stored at 4 °C.

Coating of magnetic beads and panning

Dynabeads M-270 Epoxy and Carboxylic Acid (Dyna, Invitrogen, Carlsbad, CA) were coated and blocked according to instructions from the manufacturer. Antigens were human γ -globulin, testosterone-BSA, and β -estradiol-BSA (all from Sigma, St. Louis, MO), used as stock solutions of 5 mg/mL in PBS, except for testosterone-BSA in 70% ethanol. Coating of the beads was monitored by estimating the amount of bound protein by the Micro BCA Protein Assay Kit (Pierce, Waltham, MA). The reaction was carried out with 3 to 5 µL of coated beads, and the response was compared to uncoated beads and the BSA standard curve.

Panning was performed with a magnetic bead processor KingFisher (ThermoElectron). The coated beads were incubated with 100 μL of phages for 1 h. The phage titers of the phage library stocks were 2 to 3×10^{12} colony-forming unit (cfu)/mL, and the input titers determined during the preliminary experiments were 5 to 120×10^{10} cfu/mL. The coated magnetic beads incubated with the phages were collected and resuspended every 15 min to avoid precipitation to the bottom of the well. Beads were then washed 5 times with PBST (PBS, 0.05% Tween-20) for 20 s and eluted for 15 min with 100 μL 0.1 M HCl-glycine (pH 2.2) supplemented with 0.05% Tween-20 to avoid aggregation of the beads. The eluate containing the phages was then neutralized with Tris buffer and used to infect freshly grown XL1-blue cells.

Phage growth on microplates

Of XL1-blue cells grown to $A_{600} = 0.7$ to 1, 900 μL was infected with the eluate from the previous step for 15 min without shaking at 37 °C. The infection and further cultivation steps were performed in 96-deep-well plates (ABgene, Rochester, NY). The plates were incubated in a Multitron incubator shaker (Infors, Bottmingen, Switzerland) at 30 °C and 700 rpm with 80% humidity. A sample of 5 to 50 μL of infected bacteria was plated on LB-amp plates. The residual infection was supplemented with antibiotic (20 μg carbenicillin) in 5 μL SB and grown for 1 h at 37 °C, after which more antibiotics were added (30 μg carbenicillin, 10 μg tetracyclin) in 5 μL SB. After an additional hour of cultivation, about 5×10^9 pfu VSCM13 helper phages were added with antibiotics (25 μg carbenicillin, 5 μg tetracyclin) in 20 μL SB. Infection was allowed to proceed for 15 min at 37 °C and 200 rpm, after which the speed was increased back to 700 rpm for 2 h. The antibiotic kanamycin (105 μg) was added, temperature decreased to 30 °C, and the cultivation was continued overnight. Next morning, the cells were centrifuged down for 10 min at 2000 g (Eppendorf Centrifuge 5810R). The phages were precipitated from 500 μL of supernatant as described for the library phages and resuspended in 115 μL PBS. Then, 100 μL of this suspension or 100 μL of the o/n supernatant as such without PEG precipitation was used for a subsequent round of panning. The unused phage suspension or supernatant was transferred to a new plate for short-term storage at 4 °C or -20 °C for later use.

ELISA for the eluted scFv phage pools

After 4 rounds of panning, the enrichment of the specific phages was monitored by phage ELISA. Of the phage containing supernatant 50 μL was used to infect 500 μL of XL1-blue cells grown to $A_{600} = 0.7$ to 1. Phages were grown as described previously, and the supernatant was used for ELISA. Titer was determined for the supernatants, and they were in the range of $(1-4) \times 10^9$ cfu/mL.

MaxiSorp 96C-microplate wells (Nunc, Rochester, NY) were coated with 500 ng of antigen in 100 μL PBS for 1 h at room temperature (RT). Plates were washed 3 times with 300 μL PBST and blocked with 200 μL of BSA/PBS for 30 min. After washing, 100 μL of the phage supernatant diluted 1/10 in BSA/PBS was added to the wells and incubated for 1 h on a plate shaker. After washing of the plate 3 times with PBST, a 1/5000 dilution of anti-M13-peroxidase (GE Healthcare, Fairfield, CT) in 100 μL BSA/PBS was added to the wells and incubated for 1 h at RT. Plates were washed and supplemented with 100 μL of substrate solution. The substrate solution for peroxidase was either 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) solution (1 mg ABTS, 4.5 mL 50 mM $\text{Na}_3\text{-citrate}$, pH 4, 1:583 30% H_2O_2) read at A_{415} with a Varioskan plate reader (ThermoElectron) or SureBlue 3,3',5,5'-tetramethylbenzidine (TMB) Microwell Peroxidase Substrate solution (1-component, KPL). For the latter, the color-forming reaction is quicker, so it was stopped by adding 100 μL 1M HCL, and the plates were read at A_{450} .

Production of scFv antibodies and ELISA

Colonies were picked from the LB-agar plates with QPix-colony picker (Genetix, Hampshire, UK). From each round, two 96U-well plates (Nunc, Nunc) of both kappa and lambda clones were picked and transferred to 100 μL of Master medium (SB, antibiotics, 1% glucose, 1 $\times$ FreezingBuffer²⁷ per antigen). Plates were incubated for bacterial growth overnight. Using the robotic system, plates were read at A_{630} , and 7 μL of the bacterial suspension was used to inoculate 200 μL of Induction medium (SB, antibiotics, 0.1% glucose and 1 mM isopropyl-beta-D-thiogalactopyranoside [IPTG]) for antibody production on a new 96U-well plate. Plates were incubated for bacterial growth and scFv production at 30 °C overnight. After centrifugation, the supernatant was used for ELISA.

The robotic system was used for pipetting the ELISA reactions at room temperature. MaxiSorp 96C-microplate wells (Nunc) were coated with 500 ng of antigen in 100 μL PBS for 1 h. Plates were washed 3 times with 300 μL PBST, blocked with 200 μL BSA/PBS for 30 min, and then washed again. Then, 50 μL supernatant and 50 μL BSA/PBS were added to each well, mixed, and incubated for 1 h. After washing of the plate, a 1/3000 dilution of anti-c-myc-peroxidase (Roche, Indianapolis, IN) in 100 μL BSA/PBS was added to the wells and incubated for 30 min. Plates were washed and supplemented with 100 μL SureBlue substrate solution and incubated for 30 min. The color reaction was stopped by adding 100 μL 1M HCL, and the plates were read at A_{450} .

Clones determined to be positive in primary ELISA were transferred from master plates to new mother plates using a Biomek 2000 pipetting robot. Bacteria were grown and induced as described previously. The supernatant from these second induction

Table 1. Phage Titers of the Panning with γ -Globulin

Round	Phages in Supernatant		PEG-Precipitated Phages	
	Input Titer	Output Titer	Input Titer	Output Titer
	$\times 1E10$ cfu/mL	$\times 1E4$ cfu/mL	$\times 1E10$ cfu/mL	$\times 1E4$ cfu/mL
I	270	105	—	—
II	5.2	46	25	3.0
III	13	130	40	100
IV	13	2100	120	2500

Four rounds of panning were performed with the antigen coated on epoxy-activated beads. Only the kappa scFv phage library was used. Input titer represents the concentration of multiplied phages (colony-forming units, cfu) in the supernatant used in the selection. For round I, it is the concentration of the phage library, which is the same for both unprecipitated and precipitated phages. Output titer is the concentration of the eluted phages from the selection. PEG, polyethylene glycol.

plates was used for secondary ELISA. Two plates were coated and blocked as previously described for each induction plate, one with antigen and one with BSA. Then, 20 μ L supernatant and 180 μ L BSA/PBS were mixed on the BSA-coated plate, and 100 μ L of the mixture was transferred to an antigen-coated plate. The plates were then processed as described for primary ELISA and read at A_{450} .

Fingerprinting and sequence analysis

Clones giving at least 1.4 times the signal on binding the antigen compared to BSA were considered positive, and their DNA was analyzed by BstNI-digestion. Of bacterial cell suspension 1 μ L was added to 100 μ L of water and heated at 100 °C for 5 min. The scFv coding sequence was then amplified from the plasmid released from the cells by PCR, and the product was digested with BstNI (New England Biolabs, Ipswich, MA). Fragments were run on 3% low melting point agarose gel and visualized by ethidium bromide staining. Clones giving different digestion patterns were sequenced by ABI 310 using BigDye 3.0 Sequence Termination Kit (Applied Biosystems, Foster City, CA).

Amino acid sequences were aligned with the ClustalW program, and homology trees were drawn from the outcome file with the Phylip 3.66 program available on the Internet. Families and subgroups were determined with program DNAPlot provided by the V-base site on the Internet.

RESULTS

Scaling down the enrichment of phages

Human γ -globulin was used as a model antigen for setting up the automated enrichment and screening procedures. For immobilization of the antigen, we used chemically activated magnetic beads due to the large surface area and easy processibility with a magnetic bead processor. The equipment allows automated binding and washing steps of 24 (KingFisher) or 96 (KingFisher 96) samples in parallel. Human γ -globulin coated on epoxy-activated beads was used as a model antigen

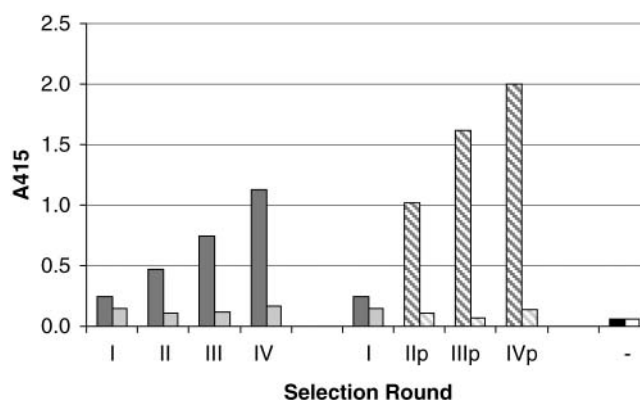


FIG. 1. Phage pool enzyme-linked immunosorbent assay (ELISA) showing enrichment of the phages in panning with γ -globulin. Darker bars represent antigen-coated wells, and lighter bars represent bovine serum albumin (BSA) background binding. Uniformly colored bars represent the unprecipitated phages, and striped bars represent the polyethylene glycol (PEG)-precipitated phages (p). Background binding without phages is marked with (—).

for scaling down the phage enrichment step. Four successive rounds of selection were performed with our naive kappa-variable regions containing scFv phage library on the microplate format. After the first selection round, half of the multiplied output phages were precipitated and used for the next round of selection in parallel with the unprecipitated supernatant. As expected, phage titers were higher when the phages were precipitated. However, even without the precipitation step, the culture supernatant still contained enough phages in 100 μ L to continue the selections successfully (Table 1). The specific enrichment can be seen in the ELISA performed with the pools of enriched phages of the same experiment (Fig. 1).

Optimization of the ELISA protocol for the robotic system

The ELISA protocol must be as simple as possible to be effectively handled by the robotic system. Coating, blocking, and

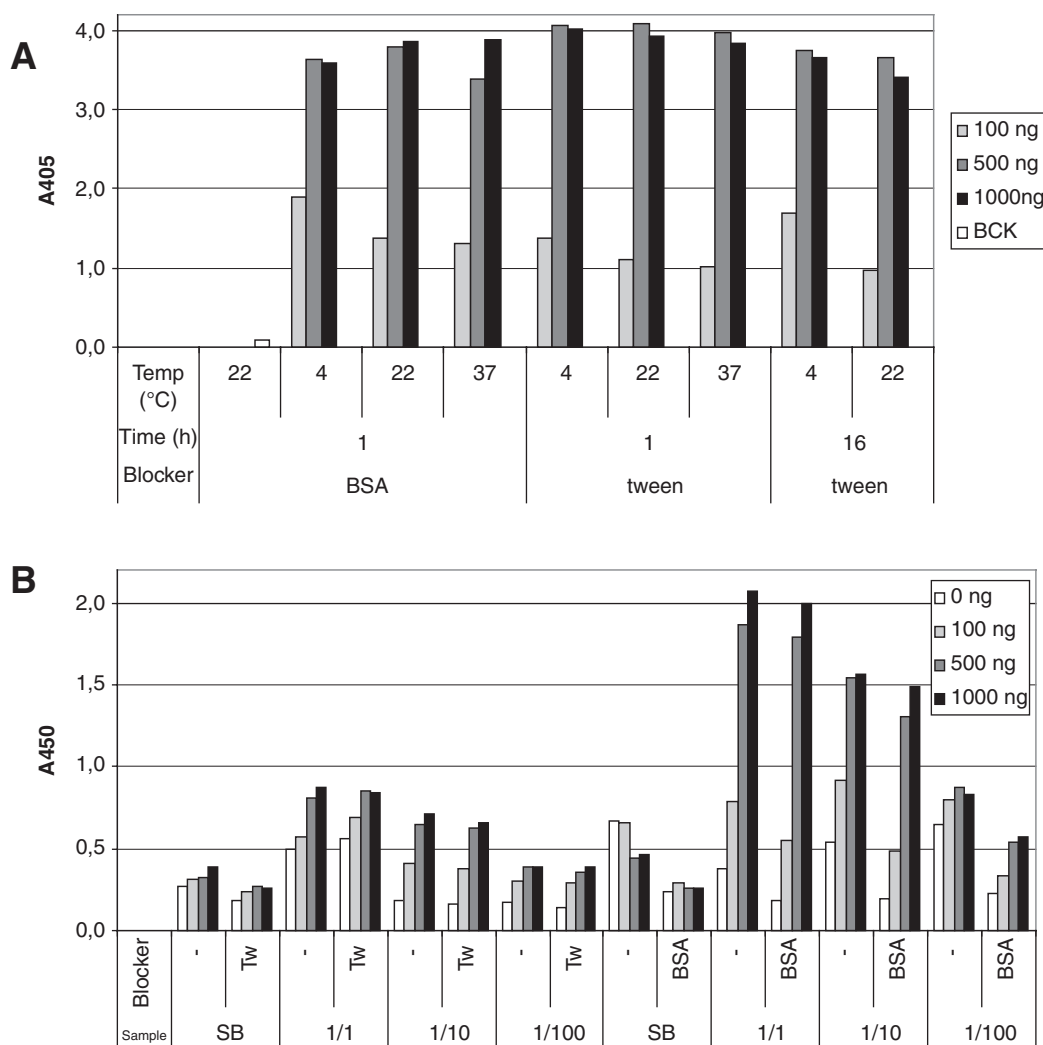


FIG. 2. Optimization of the enzyme-linked immunosorbent assay (ELISA) protocol with γ -globulin. **(A)** Coating was tested in different temperatures for 1 h or overnight (16 h). Coating was tested in different temperatures for 1 h or overnight (16 h), and 100/500/1000 ng of γ -globulin was used for coating. For blocking of the background binding, bovine serum albumin (BSA) or Tween-20 was used. Anti-myc monoclonal antibody and goat antimouse antibody conjugated with alkaline phosphatase were used for detection. **(B)** Blocking conditions were tested for anti-myc (9E10)-HRP detection antibody with TMB substrate. Wells were coated with 0/100/500/1000 ng of γ -globulin. Sample was #25 containing supernatant diluted in Superbroth (SB) medium (1/1, 1/10, or 1/100) or only SB as background control. The wells were tested for blocking with BSA or Tween-20, or they were left unblocked (–).

detection steps were optimized for human γ -globulin antigen. For coating conditions, the amount of antigen (100/500/1000 ng), coating time (1/16 h), and temperature (4/22/37 °C) were varied. For blocking, 0.5% BSA or 0.05% Tween-20 in PBS was used. The effects of different coating, blocking, and detection conditions were detected with a human γ -globulin binding scFv, previously isolated from the VTT naive scFv library (data not shown). One hour at room temperature appeared to be sufficient for coating and even better at least with the model antigen than the conventional incubation at 37 °C (**Fig. 2A**). Anti-myc (9E10) mAb and goat antimouse-AP pair were switched to direct detection with

anti-c-myc (9E10)-peroxidase conjugate. TMB was chosen as a substrate due to its higher sensitivity compared to ABTS used previously in our laboratory. With this setup, BSA blocking gave better signals (**Fig. 2B**). As a result of the optimization, it was decided to perform the coating with 500 ng of human γ -globulin in PBS for 1 h at room temperature. The wells were washed 3 times with PBST and blocked with BSA.

In the primary ELISA, 161 clones from the preliminary selections with γ -globulin were analyzed. Of these clones, 57 had 2-fold the signal of binding to γ -globulin compared to the detection background signal and were thus considered positive in the

Table 2. Output Titers of the Phages Eluted from the Testosterone-BSA and β -estradiol-BSA-Coated Beads during Selection Rounds

Round	Testosterone-BSA		β -estradiol-BSA	
	Kappa	Lambda	Kappa	Lambda
I	16	16	24	16
II	1.2	1.1	1.4	1.7
III	4.8	9.3	2.9	15
IV	44	112	120	175

Four rounds of panning were performed with kappa and lambda phage libraries. Input titers were not determined for this experiment. BSA, bovine serum albumin. Output titer $\times 10^6$ cfu/mL.

primary ELISA. Of these clones, 18 best-performing ones were analyzed in the secondary ELISA. Fifteen of them bound γ -globulin 2-fold better than the BSA-background and were thus considered positive in the secondary ELISA. Thirteen of these clones were analyzed by fingerprinting, and 9 of them seemed to be different. These γ -globulin-binding clones obtained while optimizing the automated panning procedure were not analyzed further.

Panning with 2 antigens in parallel

Testosterone-BSA and β -estradiol-BSA were used as antigens to test the performance of the developed automated selection and screening procedures. These antigens were chosen because they had been previously used in manual panning with the same antibody phage library. Carboxy- and epoxy-activated beads were tested for coating. According to the micro-BCA protein assay, the former was coated more efficiently but tended to aggregate with the steroids. It was decided to use epoxy-activated beads with the steroid conjugates. The scFv libraries with kappa and lambda light chains were panned separately. Four rounds of panning were conducted as described in Materials and Methods.

After infection of freshly grown bacteria with the eluted phages, a part of the infected bacteria was plated on selective plates and grown overnight. The next day, the number of colonies was estimated per volume of eluate to obtain the output phage titers (Table 2).

The input titers were not determined because having several antigens in parallel in the selections would be too laborious to plate output phages from all of them. Results of the phage pool ELISA (Fig. 3) show that there was specific enrichment of phages toward the antigens. The ELISA signal increased round by round. Antibodies from the lambda library were enriched more rapidly with both antigens because the signal was significantly elevated already after the second round of panning.

Screening of positive clones

Two full 96-well microplates of colonies were picked from each round of the selections with both antigens. They were grown overnight on the master plates and diluted 1/30 to induction

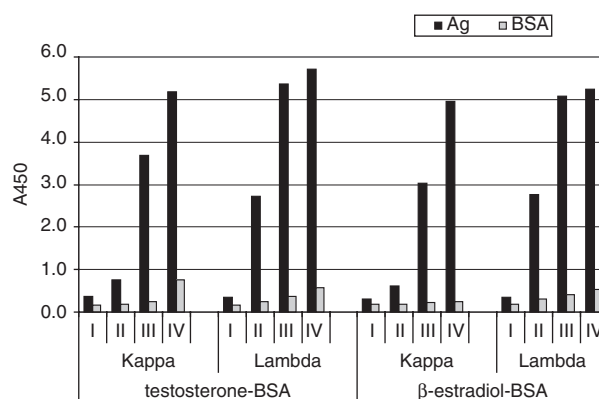


FIG. 3. Results of the enzyme-linked immunosorbent assay (ELISA) performed with the output phages eluted from the panning rounds with testosterone-bovine serum albumin (BSA) and β -estradiol-BSA. Microplate wells were coated with BSA-conjugated antigen (dark gray) or left uncoated (light gray). All wells were then blocked with BSA. The number of phages analyzed was about 3×10^5 pfu/well.

medium containing 0.1% glucose and 1 mM IPTG. The clones were incubated at 30 °C overnight to produce scFv for ELISA. The antigen binding of the scFv fragments was measured by the optimized ELISA protocol transferred to the robotic system (Fig. 4). The ORCA arm transferred the plates between the stations, where plates were washed and reagents were transferred to them (Fig. 4B). Growth of the bacteria was monitored by measuring absorbance at 630 nm. Although the growth was minimal in some wells on the master plate, there was usually a pellet after induction. If there was no pellet, the well was considered a negative sample. The cutoff level for positive clones was set to 1.7 when compared to the background signal of the negative samples.

A summary of the ELISA results (Table 3) shows that the more selection rounds were performed, the more antigen-binding clones were seen in primary ELISA. After 3 rounds of selection, half of the clones enriched with testosterone-BSA from the kappa library were positive. In the case of the lambda library, the same result was achieved only after 2 rounds of selection. With β -estradiol-BSA, 40% of the screened colonies were positive after 3 rounds of enrichment with the kappa library. Enrichment from the lambda library with β -estradiol-BSA was the best; 60% of the clones were positive after 2 rounds of panning.

The antigen-binding response of the clones considered to be positive in the primary ELISA was confirmed and compared to BSA binding in the secondary ELISA. Two plates of both testosterone-BSA and β -estradiol-BSA binding clones were picked using the Biomek 2000 pipetting station (Fig. 4A). Bacteria were grown, induced, and screened as for the primary ELISA, except that this time, the samples were pipetted onto 2 plates, one coated with the antigen and the other with BSA, and processed in parallel. Clones giving 1.4 times the response for binding to the antigen

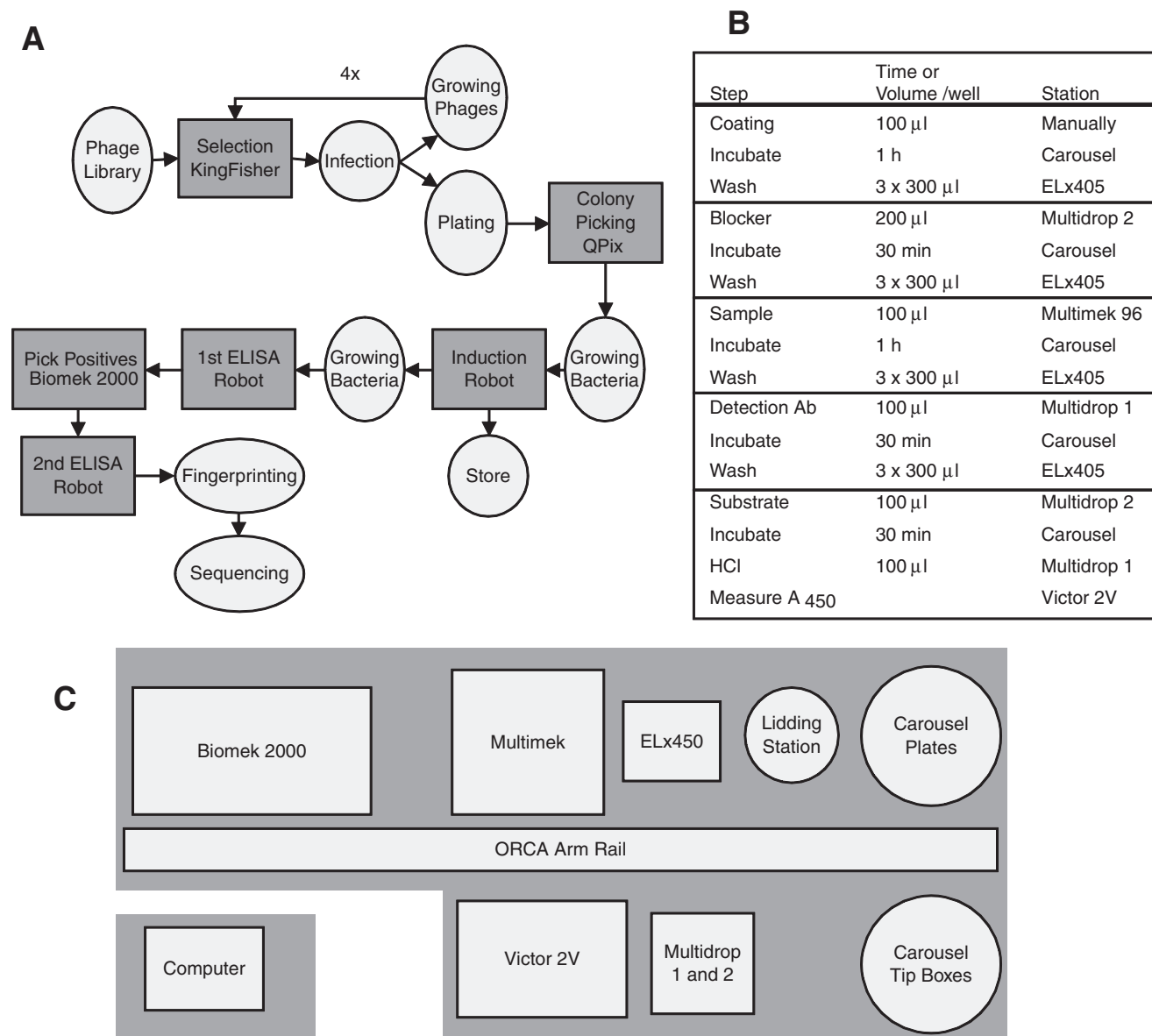


FIG. 4. Overview of the automated phage display protocol. (A) Workflow diagram of the automated panning procedure. Squared steps are performed automatically. (B) Steps of the automated enzyme-linked immunosorbent assay (ELISA) protocol performed by the robotic station. (C) Schematic view from above the robotic station.

compared to binding to BSA were considered positive. Of the testosterone-BSA and estradiol-BSA binding clones, more than half bound the BSA-conjugated antigen but not BSA from the second, third, or fourth round (Table 3).

Sequence analysis of the antigen-binding clones

The confirmed positive testosterone-BSA and β -estradiol-BSA clones were analyzed by BstNI fingerprinting analysis. The region

coding the scFv antibody fragment was amplified from a lysate of a colony by PCR and then digested with BstNI restriction enzyme. The fragments were separated on an agarose gel to be visualized and compared with each other. One plate of 96 samples was run on 2 gels. Especially with lambda clones, it was clear that most of the clones from the third and all from the fourth round had the same antibody coding sequence. This was confirmed by sequencing. Forty of both testosterone-BSA and β -estradiol-BSA binding clones were chosen for sequencing (Table 4). Homology trees

Table 3. Summary of Screening Results from Panning with Testosterone-BSA and β -estradiol-BSA

	Selection Round	Primary ELISA		Secondary ELISA	
		Clones Screened	Positive % vs. Detection	Clones Screened	Positive % vs. BSA
Testosterone-BSA					
Kappa library	I	192	4.2	16	62.5
	II	192	30.7	16	93.8
	III	192	51.6	32	81.3
	IV	192	65.1	32	65.6
Lambda library	I	192	4.7	16	50.0
	II	192	50.5	16	68.8
	III	192	84.9	32	96.9
	IV	192	99.0	32	96.9
β-estradiol-BSA					
Kappa library	I	192	2.6	16	31.3
	II	192	13.0	16	81.3
	III	192	41.7	32	81.3
	IV	192	76.0	32	71.9
Lambda library	I	192	5.2	16	68.8
	II	192	60.4	16	100
	III	192	93.8	32	96.9
	IV	192	95.3	32	96.9

The table shows how many clones were screened in the primary and secondary enzyme-linked immunosorbent assay (ELISA) from each panning round, as well as how many of them were positive, compared to detection background in the primary ELISA and to bovine serum albumin (BSA) background binding in the secondary ELISA.

Table 4. Summary of Sequenced Clones from Panning with Testosterone-BSA and β -estradiol-BSA

	Selection Round	Clones Sequenced	New Different Sequences
Testosterone-BSA			
Kappa library	I	4	4
	II	5	4
	III	9	8
	IV	9	6
Lambda library	I	4	3
	II	2	1
	III	4	3
	IV	3	0
Total		40	29
β -estradiol-BSA			
Kappa library	I	2	1
	II	6	6
	III	10	7
	IV	10	5
Lambda library	I	5	4
	II	2	0
	III	2	0
	IV	3	0
Total		40	23

The table shows the numbers of positive clones on the secondary screen from each panning round. Only a part of the positive clones was selected for sequencing, based on the fingerprinting results. The last column shows the number of clones having different sequences. BSA, bovine serum albumin.

were made from the amino acid sequences to determine which clones had an identical sequence (**Fig. 5**).

Twenty-nine of the sequenced testosterone-BSA binding clones were different (**Table 4**), and 22 clones contained kappa and 7 lambda light chain sequences (**Fig. 5C**). Only 4 of the sequenced clones occurred multiple times, the highest number being 4 identical clones. Twenty-three of the sequenced β -estradiol-BSA binding clones were different (**Table 4**), with 19 containing kappa and 4 lambda light chain sequences (**Fig. 5F**). The diversity of the β -estradiol-BSA binding clones was somewhat smaller. Five clones occurred several times, the highest number being 9 identical clones. Two of the testosterone-BSA binding clones had identical VH sequences (**Fig. 5B**), but their VL sequences varied by 6 amino acids (**Fig. 5C**). Two of the β -estradiol-BSA binding clones also had identical VH sequences (**Fig. 5E**), but one had a kappa and the other had a lambda VL chain (**Fig. 5F**).

The dominating family of VH chains of both steroid-BSA binding groups was VH3, with the dominating VL chain subgroup being VKIII (**Table 5**). There were 6 clones with the VH1 chain in both binder groups. In the β -estradiol-BSA binders, there was 1 VH4 and 1 VH6 chain. The kappa VL chains had 1 clone containing VKI and 1 clone containing VKII. Lambda clones were a little more diverse, having subgroups VL1 and VL3 but with VL5 as the most common. As expected, the greatest variability was in the complementarity determining region (CDR) HCDR3. The length of HCDR3 was 7 to 24 amino acids

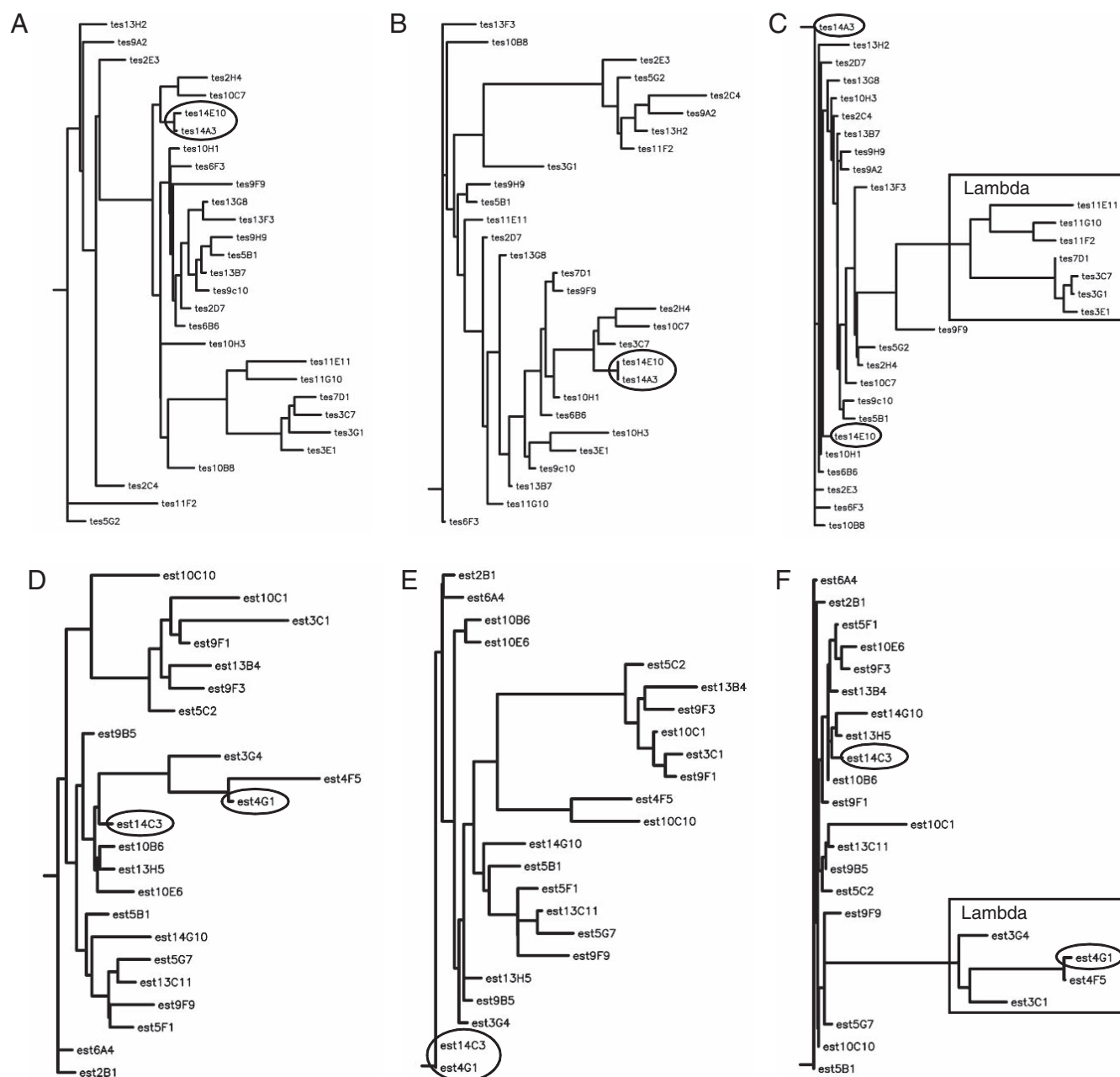


FIG. 5. Sequence homology trees of (A-C) testosterone-bovine serum albumin (BSA) and (D-F) β -estradiol-BSA binding clones. Homology trees were made with the Phylip 3.66 program with the complete scFv amino acid sequence (A, D) and with VH (B, E) and VL (C, F) amino acid sequences. The lateral distance between the clones is proportional to their homology. The circles indicate clones having identical VH chains but different VL chains.

with testosterone-BSA binding clones and 7 to 20 amino acids with β -estradiol-BSA binding clones. No consensus sequence was observed (data not shown). The other CDRs had resemblance to each other according to the family or subclass of the chain.

DISCUSSION

By optimizing the antibody phage display protocol to the microplate format, we were able to create an efficient panning

Table 5. Families and Subgroups of the Different Testosterone-BSA and β -estradiol-BSA-Binding Clones and the Selection Round from Which They Were Isolated

<i>Clone</i>	<i>Family</i>	<i>Subgroup</i>	<i>Round</i>
tes2E3	VH1	VKIII	I
tes2C4	VH1	VKIII	I
tes2H4	VH3	VKIII	I
tes2D7	VH3	VKIII	I
tes5B1	VH3	VKIII	II
tes5G2	VH1	VKIII	II
tes6F3	VH3	VKIII	II
tes6B6	VH3	VKIII	II
tes9A2	VH1	VKIII	III
tes9F9	VH3	VKI	III
tes9H9	VH3	VKIII	III
tes9c10	VH3	VKIII	III
tes10H1	VH3	VKIII	III
tes10H3	VH3	VKIII	III
tes10C7	VH3	VKIII	III
tes10B8	VH3	VKIII	III
tes13H2	VH1	VKIII	IV
tes13F3	VH3	VKIII	IV
tes13B7	VH3	VKIII	IV
tes13G8	VH3	VKIII	IV
tes14A3	VH3	VKIII	IV
tes14E10	VH3	VKIII	IV
tes3E1	VH3	VL5	I
tes3G1	VH3	VL5	I
tes3C7	VH3	VL5	I
tes7D1	VH3	VL5	II
tes11F2	VH1	VL1	III
tes11G10	VH3	VL1	III
tes11E11	VH3	VL3	III
est2B1	VH3	VKIII	I
est5B1	VH3	VKIII	II
est5F1	VH3	VKIII	II
est5C2	VH1	VKIII	II
est5G7	VH3	VKIII	II
est6A4	VH3	VKIII	II
est9F1	VH1	VKIII	III
est9F3	VH1	VKIII	III
est9B5	VH3	VKIII	III
est9F9	VH3	VKIII	III
est10C1	VH1	VKII	III
est10B6	VH3	VKIII	III
est10E6	VH3	VKIII	III
est10C10	VH4	VKIII	III
est13B4	VH1	VKIII	IV
est13H5	VH3	VKIII	IV
est13C11	VH3	VKIII	IV
est14C3	VH3	VKIII	IV
est14G10	VH3	VKIII	IV
est3C1	VH1	VL1	I
est3G4	VH3	VL1	I
est4F5	VH6	VL5	I
est4G1	VH3	VL5	I

and screening procedure to be used with several targets in parallel. Human γ -globulin was used as a model antigen to scale down the volume of phage growth and leave out the PEG

precipitation step. An efficient ELISA screening method using a robotic station was also set up and optimized. Nine different γ -globulin binding clones were found after 4 rounds of panning. The optimization of the ELISA protocol was performed using only human γ -globulin. It must be kept in mind that it might not necessarily be applicable to other antigens directly but needs further optimization for ideal blocker or detection reagents (e.g., in the situation of unspecific binding of the detection antibody to the antigen used). However, the ELISA protocol described here worked well with the steroid-BSA conjugates.

To test further the whole procedure of automated antibody generation, we panned our human naive scFv library with testosterone-BSA and β -estradiol-BSA. The outcome of the panning is shown in **Table 4**. We sequenced only about 2/5 of all of the steroid-BSA binding clones and identified 29 different testosterone-binding and 23 different β -estradiol-binding clones. Although our human naive scFv library is only modest in size (10^8 clones) compared to the largest naive libraries reported in the literature,⁴ we found more than 20 promising candidates for both of the hapten antigens.

It appears that there is no significant cross-contamination problem between the wells in the microplate conditions because only 2 of 80 sequenced clones contained more than one specimen. This was most probably due to the colony picker picking the colonies from too dense plates. Plating of the output clones is a step in the protocol that cannot be automated easily. The more antigens are screened in parallel, the more plates are needed. It is not feasible to make several dilutions of each of them but to try to estimate a dilution that is suitable for all. The performance of colony picking depends greatly on the success of the plating. If too few infected cells are plated, there are not enough colonies to be picked. On the other hand, if there are too many colonies, they are too small and dense to be picked, increasing the risk of cross-contamination.

Lou et al.¹¹ pointed out that the properties of the binders obtained from a selection depend on the selection strategy. One of the advantages of the automated system described here is that it is very flexible. Selection strategies and conditions can be altered easily. Although we do not know the detailed binding properties of the antibodies obtained here against testosterone-BSA and β -estradiol-BSA, at least the sequences are mostly different from those obtained by manual methods. Previously, β -estradiol-BSA has been used for manual panning of the same naive human scFv library (data not shown). Forty-eight kappa clones and 25 lambda clones were analyzed in secondary ELISA. Of these, 5 kappa and 5 lambda clones were sequenced. One of the lambda clones was identical to that which occurred 9 times in the clones obtained by the automated approach. These results imply that a larger number of target-specific antibodies can be enriched from the libraries with the automated method.

We have shown in this work that the throughput and efficiency of phage display can be improved significantly with the aid of automation. When the coating procedure of the antigen to the surface of magnetic beads is optimized, the

enrichment process of the phages is very efficient and easy to conduct with the magnetic bead processor. The ELISA screening with the robot is quite feasible because the screening of 12 plates takes only about 4.5 h, of which only a small part is hands-on time. The capacity of primary screening with the current equipment is 2300 clones per day. By adding 2 dispensers, this number could be doubled. The system is 384-plate format compatible, which would increase the throughput by a factor of 4. This brings the theoretical capacity of ELISA screening up to 18,400 clones per day. If 2 plates of colonies from 2 rounds of selection with kappa and lambda libraries (eight 96-well plates, 768 clones) are screened in the 384 format, colonies for 24 antigens could be screened in 1 day. Ninety-six antigens would take 4 days, and about 80 µg of antigen would be consumed. With the current throughput in 96 format, we are able to screen colonies for 3 antigens per day with about 240 µg of antigen consumed.

The bead-based screening system using the homogeneous fluorometric microvolume assay technology²³ could be a potential alternative to the ELISA-based screening system described here. The performance should be tested with a scFv format-compatible fluorescently labeled antibody. The benefits of a homogeneous assay in the screening would be indisputable, as it would save time, labor, and reagents.

As the throughput of screening increases, characterization of the selected antibodies rapidly becomes the limiting factor. The most challenging task at present is the affinity ranking of the antibody candidates. Competitive ELISA tests are often very unreliable compared to surface plasmon resonance (SPR) data.²⁸ SPR measurements, on the other hand, require the antibody to be purified, although there is one very recent report on affinity ranking of scFv fragments from crude lysates.²⁹ Two recent examples of high-throughput purification methods for antibodies exist. One uses tips filled with protein-A sepharose (not suitable for all scFvs) that can be used manually with a multichannel pipette or with a pipetting robot.²⁸ The other approach uses His-tagged antibody fragments purified by the Qiagen BioRobot 3000.³⁰ Flow cytometry-based high-throughput analysis methods are very promising, giving information of cross-reactivity and even kinetic properties of the binding events.¹⁹ High-throughput purification and characterization methods such as those described above would help to take full advantage of the throughput of the automated panning and screening system described in this article.

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