REVIEW

Automated Screening Procedure for High-Throughput Generation of Antibody Fragments

Johan Hallborn and Roland Carlsson
BioInvent Therapeutic AB (publ), Lund, Sweden

BioTechniques 33:S30-S37 (December 2002)

ABSTRACT

In the emerging field of proteomics, there is an urgent need for catcher molecules such as antibodies for detecting the proteome or parts of the proteome in a microarray format. A suitable source for providing a large diversity of binders is obtained by combinatorial libraries, such as phage display libraries of single chain antibody fragments (scFv) or Fab fragments. To find novel binders from the n-CoDeR libraries with a high throughput, we have automated the screening process with robotics. The automated system is configured to screen tens of thousands of clones per day to target antigens in various formats, including peptides and soluble proteins, as well as cell-bound targets; thus, it is well designed to meet demands from the proteomics area.

INTRODUCTION

In the era of proteomics, which has followed the genomics era, methods for simultaneously studying an abundance of proteins and protein variants in biological samples are becoming increasingly important. Such methods have applications in many areas, including drug target and disease marker identification, diagnosis of disease, and in the drug development process for evaluation of drug efficacy and toxicity, as well as in basic biological research. Today, a variety of methods including 2-dimensional (2D) gel electrophoresis in combination with mass spectrometry (MS) are widely used to analyze proteins, and as many as 2000–10 000 different proteins can be analyzed from one 2D gel (30,38). Also, 2D or 3D liquid chromatography, in combination with MS, has been employed in the study of many different proteins and protein variants (35). The major drawbacks of these technologies are that they are rather time-consuming and also require prohibitively large sample volumes. Thus, methodology, with higher throughput and less demands on amounts and volumes of material, is needed.

Recently, developments within miniaturization, signal detection, and data processing systems have allowed formation of arrays comprising a huge number of assay points. The most advanced arrays have spots with oligonucleotides or DNA and are used to assess levels of mRNA in cells and tissues (1,5,6,21). However, since mRNA levels do not directly correspond to protein levels and one mRNA can give rise to several forms of proteins, because of posttranslational modifications and differential splicing, arrays that have the capacity to analyze simultaneously an abundance of proteins and protein variants are in demand. Technology pertaining to the miniaturized format, detection systems, and data processing have been developed within the area of DNA arrays and can be applied largely for protein-based arrays. Problems that need to be specifically addressed relate to surface and binding chemistry, arraying of a multitude of discrete binding or catcher molecules, and, of course, the generation of stable binders per se. In contrast to DNA arrays, in which binding molecules may be defined by sequence and synthesized onto the surface of the array, protein-based catcher molecules with defined and predetermined specificities cannot be produced in such a way. Instead, protein-based catcher molecules need to be developed for each ligand, not knowing beforehand the sequence or structure of a good catcher molecule. Arrays can be seen as miniaturized variants of assay formats having existed for many years. Typically, such formats include enzyme-linked immunosorbent assay (ELISA) and other types of immunometric assays utilizing antibodies as catcher molecules. These antibodies may be developed through immunization of animals yielding monoclonal or polyclonal formats or may be developed using recombinant technologies using libraries of randomly recombined antibody fragments. Until now, the few protein-based arrays that have been presented have utilized monoclonal and full-length antibodies produced by conventional hybridoma technology and obtained from commercial or in-house sources. These arrays have consisted of antibodies with specificities against known antigens, e.g., cytokines (15–17,26), cell signal, or cell matrix proteins (19), but also other proteins (9), and have counted as many as 250 different antibody specificities (19). The results concerning demands on material and sensitivity are encouraging (19), but still, the number of assay point per array need to be increased in order to compete successfully with 2D gel electrophoresis.

It is anticipated that many new antibody specificities will need to be developed in order to be able to ask the pertinent questions about differential expression of antigens in relation to, e.g., target and marker identification or for analysis of effects of toxic substances or drugs on cells and tissues. Thus, there is a pronounced need to rapidly develop antibodies or binders based on other scaffolds fulfilling these criteria.

Combinatorial libraries harboring extensive variability have been used to select and identify specific binders against predetermined molecular structures. The libraries have been built using totally random peptide sequences (33) or use larger do-

mains, such as bovine trypsin inhibitor (BPTI) (22,29), or protein A (27) as scaffold structures into which variability has been introduced at defined sites.

The most commonly used protein scaffold structure is that of the antibody variable domain. Generation of variability using this scaffold has been achieved in many different ways, from random combination of heavy (VH) and light (VL) chain variable domains (23), to introduction of variability into the antibody scaffold using synthetic (14) or semisynthetic approaches (34). Formats based on the antibody scaffold include single chain antibody fragments (scFv) (14,34) or Fab fragments (8). Fragments isolated from the libraries may be used, as they are for research or analytical purposes, or be transformed into other formats suitable for therapy.

Highly diverse DNA expression libraries have been generated using combinatorial principles. The larger the library, the higher the probability will be that the library contains a specific binder to any given antigen and that this binder is of a high affinity. Expression libraries are often made at the DNA level, and the variability is then introduced into expression vectors, which are transformed into host cells, e.g., bacteria. From the first libraries, which were based on variability in antibody fragments, the synthesized antibodies were expressed in a soluble form. In order to screen these libraries, the library members were cloned and screened using filter lifts (18). This method only allowed a limited number of clones to be screened. The introduction of so called display technologies, in which the encoding genotype was physically linked to its phenotype, allowed for a much more efficient handling of large expression libraries. Various forms of display technologies using bacteria (4), yeast (2), or bacteriophage (25) have been presented, and today, so-called phage display is widely used for display of various types of binders, e.g., antibody fragments.

The most commonly used bacteriophage for phage display belong to the filamentous phage family. The best characterized are M13, fd, and f1 that infect *Escherichia coli* cells containing the F factor. The binder is displayed on the surface of the phage particle as a fusion to a coat protein, e.g., protein 3 or protein 8. The phage particle expressing the specific binder can then be isolated together with its encoding gene. If needed, the isolated phage pools may be amplified and reselected against the antigen in further rounds (25), yielding a large proportion of phage specific for the antigen.

Phage display libraries often have around 10^9 and even reaching 10^{11} members (31). In order to achieve larger libraries, alternative display methods have been developed. These approaches utilize in vitro translation and coupling of the translated protein to its encoding polynucleotide. They include various forms of ribosome display (11,12,24), relying on noncovalent linkage of the genetic information to the protein molecule. In other display formats, which also rely on in vitro translation, there is a covalent linkage between the genetic information and the potential binding protein molecule, such as the PROfusion (37). Libraries constructed using in vitro translation approaches have a potential to yield $>10^{12}$ binders in a relatively small volume. Problems associated with ribosome display concern the stability of the complex between the mRNA and the translated polypeptide, and even if high affinity binders have been selected using this technology, it does not seem to have been taken into wide practice.

Obviously, these various formats for binders and libraries provide a rich source for selection of specific binders to be used in various aspects of the proteomics field. The challenge, thus, is to efficiently and robustly use them in selection and screening processes for generation of high quality binders at a high-throughput.

Target antigens to be used in the selection processes may be provided in different formats, including antigens purified from natural or recombinant sources, as antigen mixtures, or antigens expressed from cDNA libraries (3). The demand for high-throughput implies that several antigens need to be possible to process in parallel through the whole procedure of selection, screening, and validation of selected binders. An initial selection may often be performed using mixtures of antigen, provided that these antigens carry the same affinity tag and can be handled in a similar way. The resulting phages are then amplified and selected against the antigen in several additional rounds to achieve a large enrichment of specific binders. In these amplification–selection rounds, the antigens must, however, be handled in parallel, but separately, to prevent a limited set of binders against some of the antigens from dominating, skewing the variability in a unacceptable way. To achieve this, separate antigens may be bound to solid surfaces, like pins or magnetic beads, and handled in parallel in an automated fashion (36). The further process, including the capture and harvest of the antigen–phage complex, the rescue of phages after selections, and the subsequent amplification of enriched binders, may be scaled down to microtiter format. Further, the final removal of the phage gene fusion, to allow the expression of the binder in free form, may also be performed in a 96-well format using a thermal cycler for restriction enzyme digest, ligation, and transformation into chemocompetent bacteria.

The use of a microplate format throughout the process enables the handling of many antigens in parallel already by manual performance, and automation of the process further improves throughput. In reality, it is our experience that the limiting step is the timed access to a large number of antigens possessing the same tag, e.g., biotin, and also to some extent the manual spreading of transformed bacteria on agarose plates to achieve single clones.

From the selection procedure, a multitude of potential binders to each antigen will be provided, and these potential binders now need to be separated from each other by cloning and screened in order to find the ones with the best properties. Automation concerning colony picking, expression, and detection, as well as efficient data handling is, of course, imperative. Also different formats for screening, including screening against antigen bound to microtiter wells in the 96- or 384-well format, or imprint of either antibody fragments or antigens on membranes (4a,13) allow a large number of clones to be tested efficiently. Recently, panning of phage antibodies directly on 1D and 2D Western blots were demonstrated, suggesting an interesting approach for simultaneous selection of a multitude of phage specificities (20). Another way of increasing throughput in the screening process may be to let identifiable micro- or nanobeads coated with different antigens be subjected to the same potential binder. Thus, beads of different color composition that are coated with different antigens may be dispensed together in the same volume and screened for binders. An added benefit is that specificity and cross-reactivity may be tested for at the same time. Examples of such systems under development include Quantum Dot (Hayword, CA, USA) (7,10) and Luminex

(Austin, TX, USA) (28,32), and the multiplexity they allow may well exceed 100 different antigens in the future.

We have automated the selection and screening process in order to efficiently select and identify specific scFv and Fab fragments from our n-CoDeR™ libraries. These libraries comprise fully human antibody fragments that are displayed on M13 phage particles (34). Selections against several antigens from the libraries are routinely made in parallel by the same operator. The bottleneck is not found in the selection part of the process, but rather in the cloning, expression, and screening steps of the selected potential binders. The automated process is, therefore, focused at these

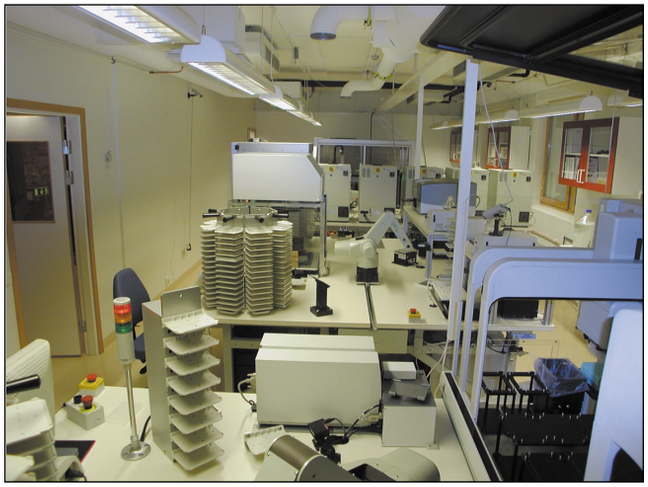

Figure 1. Robotic workstations configured to automate colony picking, bacterial expression, ELISA analysis of expressed antibody fragments, and cherry picking of actives from primary screening.

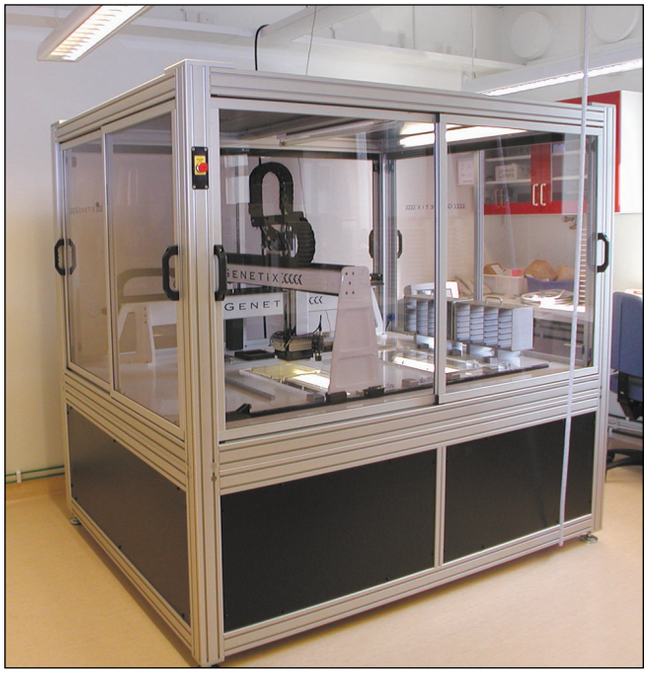

Figure 2. The Qbot workstation configured for bacterial colony picking and transfer to 96- or 384-well plates for individual clone expansion.

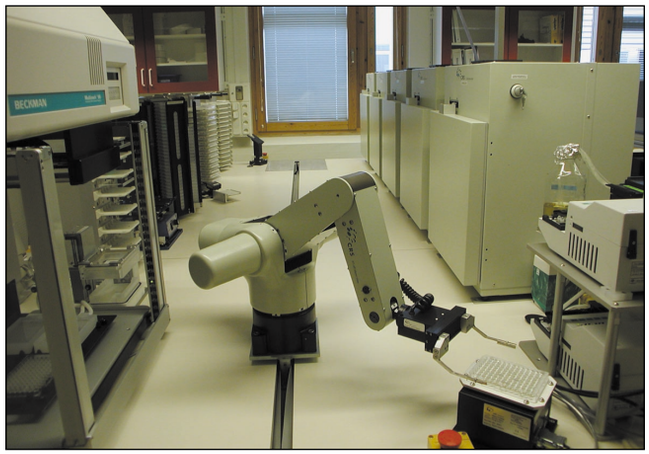

Figure 3. Robotic workstation for protein expression from bacterial cultures. Clones in 100 expression plates are simultaneously grown and expressed using incubators holding up to 20 plates each. Keeping the size limited to 20 plates per incubator made it possible to have vigorous shaking not possible in a carousel-type of incubator. Both the temperature and humidity of the incubators are restored back to normal within 1 minute after a plate has been placed or taken out from the incubator. Thus, optimal conditions for bacterial growth and expression are maintained during the process.

Figure 4. Robotic workstation for ELISA analysis of expressed clones. The system is equipped with a refrigerator for storage of antigen-coated assay plates, a multimek for transfer of scFv from expression plates to assay plates, multidrop units (Thermo Labsystems, Vantaa, Finland) for dispensing of reagents, plate washers (ELx405; Bio-Tek, Vermont, USA), and finally, an Ultra for reading the plates.

latter steps. A description follows of the integrated system with its subsystems employed in our laboratory for high-throughput generation of antibody-based binders.

RESULTS AND DISCUSSION

To have the capacity to screen tens of thousands of clones per day, we have integrated robotic workstations for expressing bacterial clones and analysis of the expressed antibody fragments. This capacity can be used to screen an extended number of clones per antigen or a high number of antigens with a lower number of clones per antigen.

The use of a robotic screening environment has significantly improved both the speed and the success rate of finding new binders in the form of scFv or Fab, to different targets (antigens). A larger number of targets, as well as more clones per target are now screened, thus giving more hit clones with different specificities–sequences and an overall higher throughput. Errors during run are now minimal because of the computer-controlled process. Clone logistics are controlled by barcoded plates and a data management system, thus securing hit clone identification and retrieval in the later stage of the automated process. The data management system has also improved the decision making of selecting hits from raw data of the primary screening to the secondary screening and sequence analysis.

The automated procedure for screening antibody fragments is divided into four subsystems. Each system is operating independently to the other systems (Figure 1). Having independent systems (robotic workstation approach), also enables freedom to branch the screening process to other detection technologies outside the automated screening process, i.e., expressed clones are diverted to fluorometric microvolume assay technology (FMAT) detection or spotted on microchips. All subsystems are designed for nonattended use once they are in operation. An alert system has been developed in-house for notifying the operator if an error occurs during run (errors in the log-file are transferred to a mobile phone as a short messaging service). Each screening campaign may span over several days. However, the independent subsystems allow several campaigns to be scheduled in parallel, giving a maximal throughput of approximately 25 campaigns per month, and up to 20 different targets, provided they have similar physicochemical characteristics, may be scheduled in each campaign.

The first system is an automated colony picking system (Qbot; Genetix, Hampshire, UK), where bacterial colonies grown on 22 × 22 cm agar plates are transferred to either 96- or 384-well plates (i.e., master plates) (Figure 2). The picking head of the Qbot has a digital camera for imaging the agar plates and 96 independent pins for picking clones. By the use of the image analysis software, bacterial colonies are identified and mapped for picking by size, roundness, and contrast. For every picking cycle, the picking head is sterilized in 70% ethanol and dried to avoid cross-contamination between clones. The capacity is more than 18 000 colonies in a 6-hour period. After picking, the Master plates are incubated overnight for growth at 37°C before transfer to the next system.

The second system is an automated expression system from Thermo CRS (Burlington, Ontario, Canada) where master plates generated in the colony picking system are replicated to expression plates for growth, expression of antibody fragments, and harvest of supernatants (Figure 3). Both the colony picking and expression systems have a high fidelity, less than 1 out of 384 clones is not growing or is cross-contaminated with other clones. Close to 40 000 clones can be processed during a 24-hour period using the 384-well format. Growth of bacteria and expression of soluble antibody fragments is performed in custom-made shake incubators (Thermo CRS) at 37°C, where vigorous shaking (600 rpm) can be performed in an on-line system. The incubators are also equipped with active humidifying devices in order to avoid edge effects (i.e., drying of peripheral wells). Clones expressed in either 96-well format or 384-well format grow to similar high cell densities and expression levels (1–10 μg/mL of antibody fragments in the supernatant). For the primary screening, where cells are expressed in a 384-well format, no sepa-

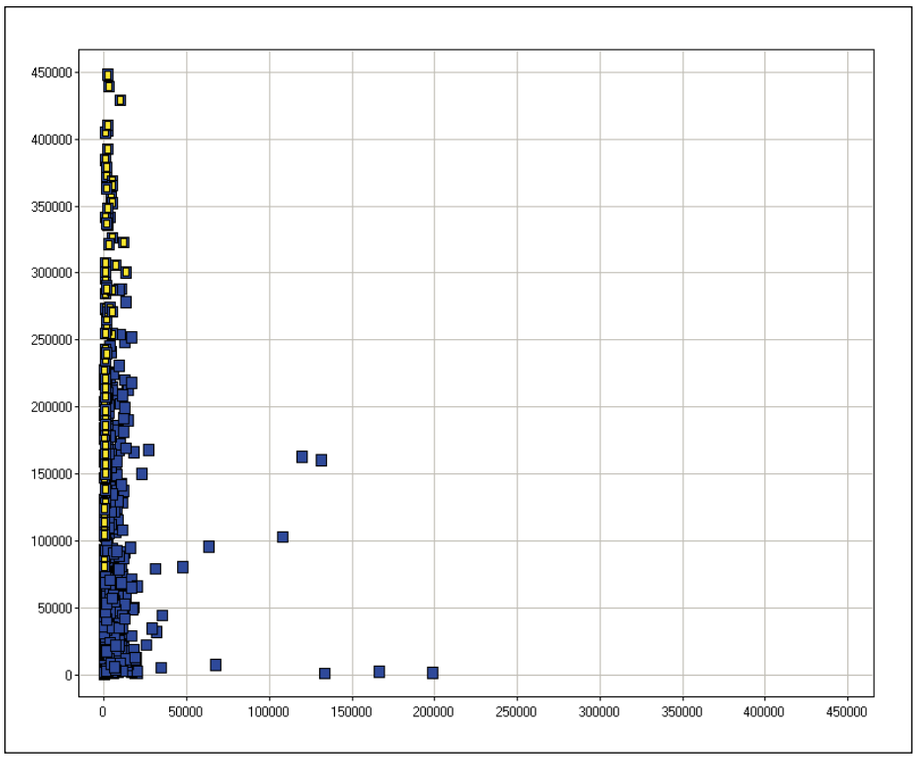

Figure 5. Graphic visualization of ELISA data from primary screening using Spotfire. Y-axis shows ELISA signals from target peptide, and X-axis shows ELISA signals from nontarget peptide. Here the best 96 clones (yellow dots) out of 1152 clones were selected as actives.

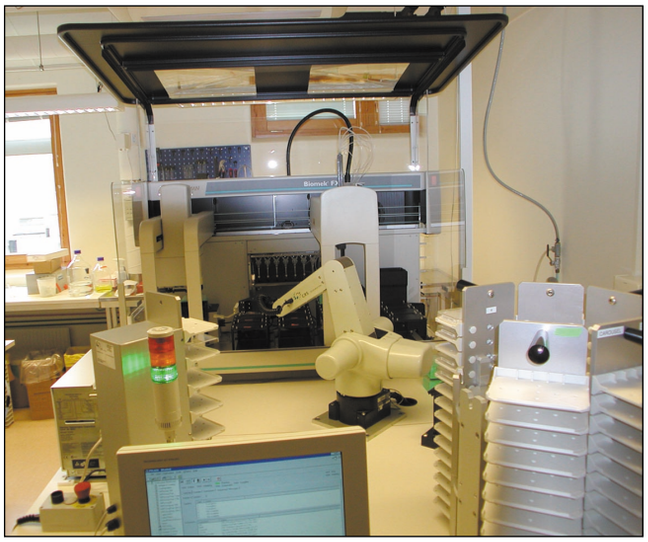

Figure 6. Robotic workstation for plate preparation (e.g., barcoding of plates) and cherry picking of actives from primary screening. A Biomek F/X on the system is configured for cherry picking using 8 independent tips. Picking of 96 actives takes approximately 3 hours.

ration of supernatant from bacterial debris, except sedimentation, is performed in the expression plate. The potential presence of bacterial debris in the supernatants from the 384-well format does not compromise the screening results, provided that the supernatants are screened for binders within 3 days. Expression of clones for secondary screening is performed in a 96-well format, and, since supernatants from these plates may, in some cases, be stored for retesting over several days, they are filtered on-line in a vacuum-driven filtration unit.

The third system is an automated ELISA system (Thermo CRS) where supernatants produced in the expression system are analyzed for binding properties (Figure 4). The ELISA system also runs with a high fidelity. The Z′ factor (a measurement of the robustness of an assay) (39) of the control plates gives a value of 0.5–1.0, which is a prerequisite for a successful high-throughput screening assay. The ELISA assay is rather complex, involving many steps. The first steps include addition of blocking buffer and transfer of supernatants from the expression plates to antigen-coated plates (prepared in advance on the cherry picking system, described below), followed by addition of secondary reagents with incubations and washing steps between all reagent additions (a total of 3 washing steps). After the last wash step, luminescent reagent is added before reading using a multi-analysis instrument (Ultra; Tecan, Maennedorf, Switzerland). Three control plates containing positive and negative controls are scheduled together with all sample plates in order to control quality of the screening process. In spite of the rather complex assay, the system has the capacity to process 10 000 clones with data from target and nontarget, thus generating 20 000 data points per day (Figure 5). The ELISA system is not limited for running ELISA assays, but also other types of assays, e.g., fluorescence polarization assays (measurement of size shift of unbound versus bound labeled peptide to binder) can be used. This latter format has a higher throughput, since it is performed as a ho-

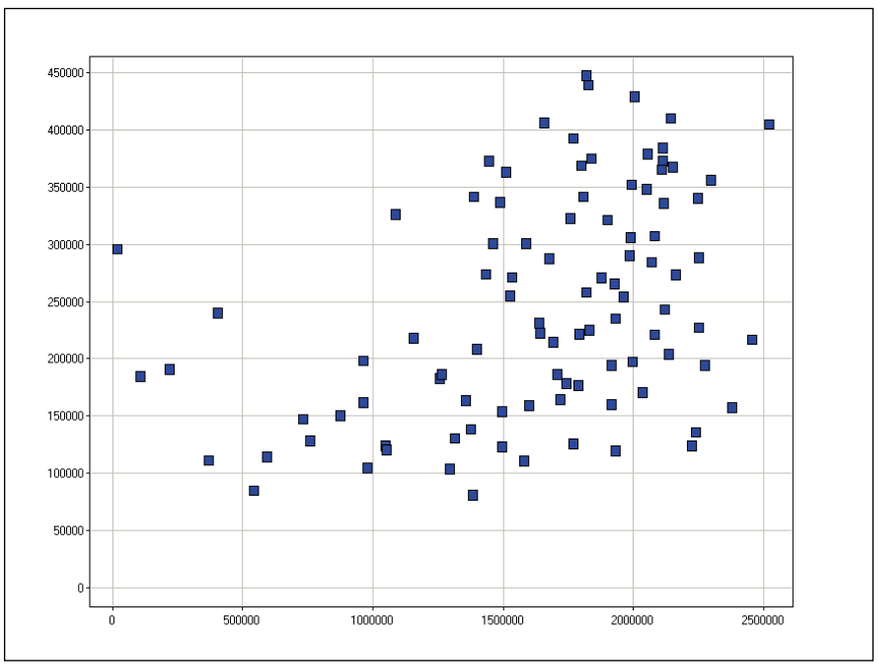

Figure 7. Hit confirmation using the Spotfire dot plot graphics. Y-axis shows actives from primary screening, and X-axis shows results from retest analysis. The active clones were reexpressed before the retest analysis in order to eliminate clones with unstable expression.

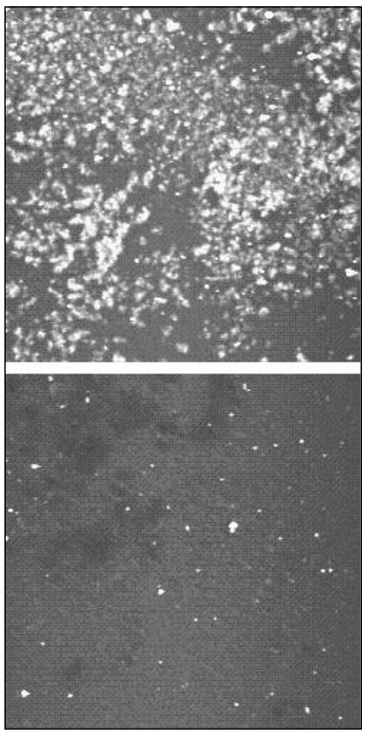

Figure 8. Images from an FMAT screening showing an active scFv binding to target cells but not nontarget cells. Assay conditions were 10 000 cells/well, 10 µL scFv supernatant, 0.1 µg mouse-anti-His antibody (R&D Systems, Minneapolis, MN, USA), 0.06 µg goat-anti-mouse antibody conjugated with Cy™5 (Amersham Biosiences, Uppsala, Sweden) and an incubation time of 4 hours before reading.

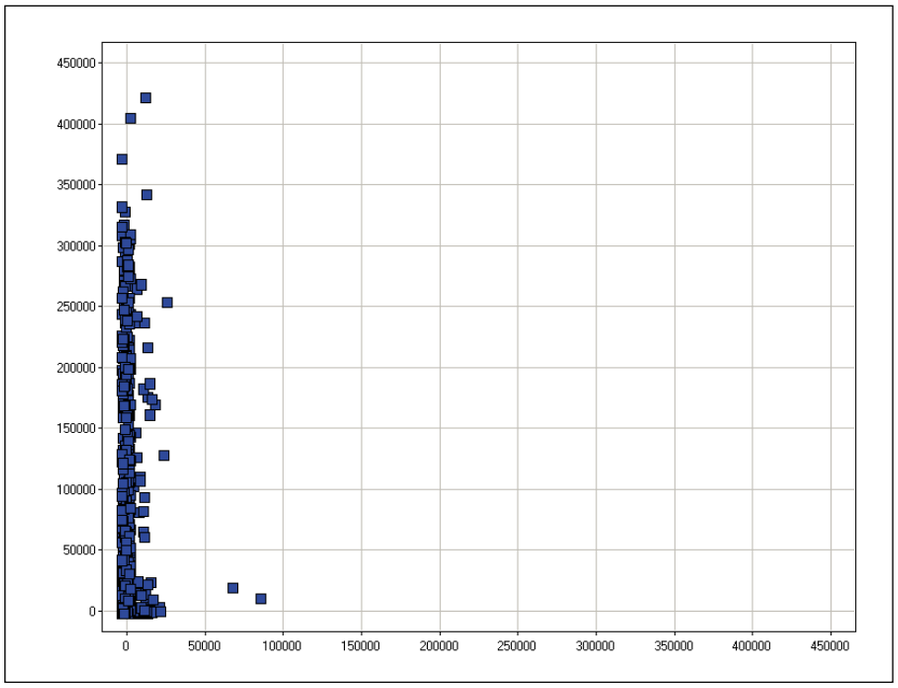

Figure 9. Spotfire analysis of screening data, derived from the FMAT instrument. Y-axis shows signals from target cells, and X-axis shows signals from nontarget cells. Data presented are from an experiment in which scFv directed against antigen differentially expressed between two cell populations were selected.

mogenous "mix and read" assay. Throughput in the assay step of the process is further increased by branching to other detection systems such as the FMAT (see below).

The fourth system is a cherry picking system (Thermo CRS) (Figure 6), where "actives" from the primary screening are collected into new master hit plates for a subsequent reanalysis (secondary screening) on the ELISA system. The cherry picking system has a cherry picking list as an input file (generated after analysis of primary screen) for transfer of the best 96 actives from the master plates, using the Biomek F/X (Beckman Coulter, Fullerton, CA, USA) pipeting station. Generated master hit plates are kept at -80°C for long-term storage, where each well is over-layered with glycerol.

Each screening campaign generates an abundance of data points. A semiautomated pipeline for data management has, therefore, been set up. The application merges screen-data with barcoded plates and the user register meta data (data describing data), such as project Identity, antigen Identity, and coating procedure. Data are then exported to Spotfire™ (Spotfire, Gothenburg, Sweden), which is a graphical data visualization tool. By using different visualization views, data points with systematic errors are rejected (e.g., data from wells with pipeting errors). Validated data are imported back to the database where percent binding and specific binding is calculated based on validated sample and control data. Processed data are then exported for analysis (ranking of clones) using Spotfire™. Actives–hits are imported back to the database, and a list of selected clones is generated for the cherry picking system.

Actives collected on a master hit plate are subjected to a retest, confirming the primary screening results (Figure 7). Clones are regrown, expressed, and analyzed again (with double samples). Usually the retest is combined with a secondary screening, where more nontargets are tested and/or a dose response is performed.

After retest and secondary screening, confirmed actives (hits) are sequenced to obtain unique hit clones. Up to 96 hits are sequenced by colony PCR and dye termination cycle sequencing, using the ABI PRISM™ 3100 DNA Analyzer (Applied Biosystems, Warrington, UK). An in-house application has been developed for making contigs and multiple alignments of the sequenced clones in an automated fashion. Selected hit clones are then characterized as purified antibody fragments in the down stream process.

For screening campaigns aiming for binders to cellular targets, the expressed clones are diverted to an off-line FMAT instrument (Applied Biosystems) for analysis. The FMAT, which is equipped with plate stackers, has a capacity to analyze cells in 96- or 384-well plates. Typically one 384-well plate takes 20 minutes to analyze, giving a throughput of 20 000 data points per day. The principle of the FMAT technology is that fluorescent-labeled cells (or beads) located at the bottom of the well are scanned with a confocal laser at a resolution of 2 μm (scan area of 1 × 1 mm), and emitted light is collected, giving an image of the cells (Figure 8). The FMAT image analysis software then calculates the number and the intensities of the stained cells (Figure 9). With this technology, screening on cells is now performed with a much higher reliability and speed compared to conventional cell-ELISA, which often gives results with great variation due to differences in cell numbers between wells. Also, the avoidance of washing steps using the FMAT contributes to speed.

CONCLUSION AND FUTURE PROSPECTS

We have established an automated screening process for identification of novel binders from the n-CoDeR libraries. The process can handle target antigens in various formats including peptides, soluble proteins, as well as cell-bound targets at a high-throughput, and is well designed to meet demands from the proteomics area. The automated system is configured for 96- or 384-well plate formats and can be used to screen, in a highly parallel manner, against a high number of target antigens per month. In order to further improve throughput, screening using a chip format is now under development. Several of the subsystems, i.e., the colony picking, expression, and cherry picking systems in the existing process, are readily adaptable to this format. In particular, for applications within antibody-based protein arrays, the chip-based format will be highly appropriate.

REFERENCES

1. **Alon, U., N. Barkai, D.A. Notterman, K. Gish, S. Ybarra, D. Mack, and A.J. Levine.** 1999. Broad patterns of gene expression revealed by clustering analysis of tumor and normal colon tissues probed by oligonucleotide arrays. Proc Natl. Acad. Sci. USA *96*:6745-6750.
2. **Boder, E.T., K.S. Midelfort, and K.D. Wittrup.** 2000. Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. Proc. Natl. Acad. Sci. USA *97*:10701-10705.
3. **Cahill, D.J.** 2001. Protein and antibody arrays and their medical applications. J. Immunol. Methods *250*:81-91.
4. **Daugherty, P.S., G. Chen, M.J. Olsen, B.L. Iverson, and G. Georgiou.** 1998. Antibody affinity maturation using bacterial surface display. Protein Eng. *11*:825-832.

4a. **de Wildt, R.M., C.R. Mundy, B.D. Gorick, and I.M. Tomlinson.** 2000. Antibody arrays for high-throughput screening of antibody-antigen interactions. Nat. Biotechnol. *18*:989-994.

5. **Ek, S., C.M. Hogerkorp, M. Dictor, M, Ehinger, and C.A. Borrebaeck.** 2002. Mantle cell lymphomas express a distinct genetic signature affecting lymphocyte trafficking and growth regulation as compared with subpopulations of normal human B cells. Cancer Res. *62*:4398-4405.
6. **Glynne, R., G. Ghandour, J. Rayner, D.H. Mack, and C.C. Goodnow.** 2000. B-lymphocyte quiescence, tolerance and activation as viewed by global gene expression profiling on microarrays. Immunol. Rev. *176*:216-246.
7. **Goldman, E.R., E.D. Balighian, H. Mattoussi, M.K. Kuno, J.M. Mauro, P.T. Tran, and G.P. Anderson.** 2002. Avidin: a natural bridge for quantum dot-antibody conjugates. J. Am. Chem. Soc. *124*:6378-6382.
8. **Griffiths, A.D., S.C. Williams, O. Hartley, I.M. Tomlinson, P. Waterhouse, W.L. Crosby, R.E. Kontermann, P.T. Jones, et al.** 1994. Isolation of high affinity human antibodies directly from large synthetic repertoires. EMBO J. *13*:3245-3260.
9. **Haab, B.B., M.J. Dunham, and P.O. Brown.** 2001. Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions. Genome Biol. *2*:RESEARCH0004.
10. **Han, M., X. Gao, J.Z. Su, and S. Nie.** 2001. Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. Nat. Biotechnol. *19*:631-635.
11. **Hanes, J. and A. Pluckthun.** 1997. In vitro selection and evolution of functional proteins by using ribosome display. Proc. Natl. Acad. Sci. USA *94*:4937-4942.
12. **He, M. and M.J. Taussig.** 1997. Antibody-ribosome-mRNA (ARM) complexes as efficient selection particles for in vitro display and evolution of antibody combining sites. Nucleic Acids Res. *25*:5132-5134.
13. **Holt, L.J., C. Enever, R.M. de Wildt, and I.M. Tomlinson.** 2000. The use of recombinant antibodies in proteomics. Curr. Opin. Biotechnol. *11*:445-449.
14. **Hoogenboom, H.R. and G. Winter.** 1992. By-passing immunisation. Human antibodies from synthetic repertoires of germline VH gene segments rearranged in vitro. J. Mol. Biol. *227*:381-388.
15. **Huang, R.P.** 2001. Detection of multiple proteins in an antibody-based protein microarray system. J. Immunol. Methods *255*:1-13.
16. **Huang, R.P.** 2001 Simultaneous detection of multiple proteins with an array-based enzyme-linked immunosorbent assay (ELISA) and enhanced chemiluminescence (ECL). Clin. Chem. Lab. Med. *39*:209-214.
17. **Huang, R.P., R. Huang, Y. Fan, and Y. Lin.** 2001. Simultaneous detection of multiple cytokines from conditioned media and patient's sera by an antibody-based protein array system. Anal. Biochem. *294*:55-62.
18. **Huse, W.D., L. Sastry, S.A. Iverson, A.S. Kang, M. Alting-Mees, D.R. Burton, S.J. Benkovic, and R.A. Lerner.** 1989. Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. Science *246*:1275-1281
19. **Knezevic, V., C. Leethanakul, V.E. Bichsel, J.M. Worth, V.V. Prabhu, J.S. Gutkind, L.A. Liotta, and P.J. Munson.** 2001. Proteomic profiling of the cancer microenvironment by antibody arrays. Proteomics *1*:1271-1278.
20. **Liu, B., L. Huang, C. Sihlbom, A. Burlingame, and J.D. Marks.** 2002. Towards proteome-wide production of monoclonal antibody by phage display. J. Mol. Biol. *315*:1063-1073.
21. **Lockhart, D.J. and E.A. Winzeler.** 2000. Genomics, gene expression and DNA arrays. Nature *405*:827-36.
22. **Markland, W., B.L. Roberts, M.J. Saxena, S.K. Guterman, and R.C. Ladner.** 1991. Design, construction and function of a multicopy display vector using fusions to the major coat protein of bacteriophage M13. Gene *109*:13-19.
23. **Marks, J.D., H.R. Hoogenboom, T.P. Bonnert, J. McCafferty, A.D. Griffiths, and G. Winter.** 1991. By-passing immunization. Human antibodies from V-gene libraries displayed on phage. J. Mol. Biol. *222*:581-97.
24. **Mattheakis, L.C., R.R. Bhatt, and W.J. Dower.** 1994. An in vitro polysome display system for identifying ligands from very large peptide libraries. Proc. Natl. Acad. Sci. USA *91*:9022-9026.
25. **McCafferty, J., A.D. Griffiths, G. Winter, and D.J. Chiswell.** 1990. Phage antibodies: filamentous phage displaying antibody variable domains. Nature *348*:552-554.
26. **Moody, M.D., S.W. Van Arsdell, K.P. Murphy, S.F. Orencole, and C. Burns.** 2001. Array-based ELISAs for high-throughput analysis of human cytokines. BioTechniques *31*:186-190, 192-194.
27. **Nord, K., E. Gunneriusson, J. Ringdahl, S. Stahl, M. Uhlen, and P.A. Nygren.** 1977. Binding proteins selected from combinatorial libraries of an alpha-helical bacterial receptor domain. Nat. Biotechnol. *15*:772-777.
28. **Oliver, K.G., J.R. Kettman, and R.J. Fulton.** 1998. Multiplexed analysis of human cytokines by use of the FlowMetrix system. Clin. Chem. *44*:2057-2060.
29. **Roberts, B.L., W. Markland, K. Siranosian, M.J. Saxena, S.K. Guterman, and R.C. Ladner.** 1992. Protease inhibitor display M13 phage: selection of high-affinity neutrophil elastase inhibitors. Gene *121*:9-15.
30. **Roepstorff, P.** 2000. MALDI-TOF mass spectrometry in protein chemistry. EXS. *88*:81-97.
31. **Sblattero, D. and A. Bradbury.** 2000. Exploiting recombination in single bacteria to make large phage antibody libraries. Nat. Biotechnol. *18*:75-80.
32. **Seideman, J. and D. Peritt.** 2002. A novel monoclonal antibody screening method using the Luminex-100 microsphere system. J. Immunol. Methods *267*:165
33. **Smith, G.P.** 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science *228*:1315-1317.
34. **Soderlind, E., L. Strandberg, P. Jirholt, N. Kobayashi, V. Alexeiva, A.M. Aberg, A. Nilsson, B. Jansson, et al.** 2000. Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries. Nat. Biotechnol. *18*:852-856.
35. **Wall, D.B., M.T. Kachman, S.S. Gong, S.J. Parus, M.W. Long, and D.M. Lubman.** 2001. Isoelectric focusing nonporous silica reversed-phase high-performance liquid chromatography/electrospray ionization time-of-flight mass spectrometry: a three-dimensional liquid-phase protein separation method as applied to the human erythroleukemia cell-line. Rapid Commun. Mass Spectrom. *15*:1649-1661.
36. **Walter, G., Z.Konthur, and H. Lehrach.** 2001. High-throughput screening of surface displayed gene products. Comb. Chem. High Throughput Screen. *4*:193-205.
37. **Weng, S., K. Gu, P.W. Hammond, P. Lohse, C. Rise, R.W. Wagner, M.C. Wright, and R.G. Kuimelis.** 2002. Generating addressable protein microarrays with PROfusion covalent mRNA-protein fusion technology. Proteomics *2*:48-57.
38. **Wildgruber, R., A. Harder, C. Obermaier, G. Boguth, W. Weiss, S.J. Fey, P.M. Larsen, and A. Gorg.** 2000. Towards higher resolution: two-dimensional electrophoresis of *Saccharomyces cerevisiae* proteins using overlapping narrow immobilized pH gradients. Electrophoresis *21*:2610-2616.
39. **Zhang, J.-H., T.D. Chung, and K.R. Oldenburg.** 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomol. Screen. *2*:67-73.

Address correspondence to:

Dr. Roland Carlsson
BioInvent Therapeutic AB (publ)
Sölvegatan 41
SE-223 70 Lund, Sweden
e-mail: roland.carlsson@bioinvent.com