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Screening isolates from antibody phage-display libraries

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Antibody phage display, coupled with automated screening, facilitates and potentiates the mining of complex combinatorial libraries and the identification of potent drug leads. In managing phage screening data, the behavior of individual phage isolates in binding assays must be linked to their antibody identities as deduced from DNA sequencing. Reviewed here are recently reported approaches for high-throughput screening of clones isolated from phage antibody libraries after selection on a defined antigen. Specific information management challenges, and possible solutions, are described for organizing screening data to enable rapid lead discovery using these antibody libraries.

Introduction

Since the first report describing M13 phage display of antibody (Ab) fragment libraries over 15 years ago [1], the approach has matured into a widely used technology platform for rapid identification of Abs to targets with therapeutic, diagnostic and research reagent applications (reviewed in References [2–4]). Humira, a fully human Ab discovered with phage display [5,6] has been approved for therapeutic use in humans, and at least 14 human Abs derived from phage display are currently in clinical trials for a wide range of human diseases [4,7,8]. Phage-display libraries are an important source of therapeutic antibodies [9] because they allow highly controlled selections to be performed *in vitro* and because fully human Ab libraries can be developed [10,11]. These libraries have been engineered to display the variable, antigen-binding domains of antibodies, either as single chain (scFv) [12] or assembled heavy and light chain (Fab) [13] fragments. Antibody variable sequences can be captured from the natural immunoglobulin gene repertoire diversity in human B-cells [14–17]. Alternatively, Ab libraries can be built using fully synthetic diversity [18–20] or by combining both natural and synthetic diversity [21], after design of diversification schemes using one [19,21] or multiple [18,20] antibody frameworks as a structural scaffold.

Desired clones are selected from a library by iterative rounds of target binding and phage amplification [4,22,23]. Successive selection rounds reduce the antibody diversity and can enrich the recovered pool in high-affinity clones. However, this may occur at the expense of clones with preferred functional properties and may limit the number of epitopes recognized by the selected antibody repertoire. To increase the probability of recovering antibodies with both high affinity and desired functionality, it is preferable to limit the number of selection rounds (e.g. to two or three) and utilize high-throughput screening (HTS) to identify the desired library members from a selected population of 1000–10,000 (or more). Rapid identification of the best leads from the screening process depends on reliable and efficient data management and analysis. This review describes reported procedures for high-throughput screening of isolates from antibody phage-display libraries and describes informatic considerations and approaches for managing the resulting data.

Antibody library screening approaches

Antigens from complex mixtures have been used to select antibodies from antibody phage-display libraries [24,25]. More commonly, purified proteins or cells with high-copy surface antigens are used to obtain binders, and a focus during the past decade has been to develop high-throughput screening methods for a large number of clonal isolates selected from a library. These screening approaches can be divided into membrane-based, microarray and microtiter/immunoassay (ELISA)-based methods (Fig. 1).

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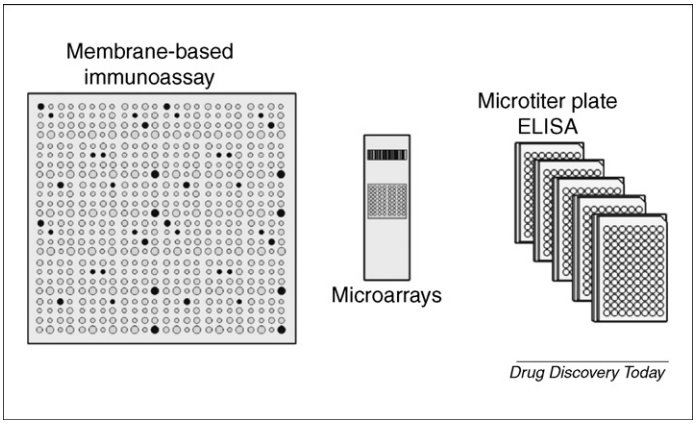

FIGURE 1

Library screening formats. Membrane-based immunoassay, microarray and microtiter plate ELISA formats have been utilized for antibody library screening. Membrane and microarray approaches allow higher density formats and advanced detection modes. Microplate-based ELISA has been most extensively used for general screening during the past decade.

Membrane-based screening

Membrane-based screening of clonal isolates orignated with hybridoma work in the laboratory of Milstein and coworkers [26] and was extended by the lab of Skerra [27,28] and later by Watkins et al. [29] by prior adsorption of capture antigen on membrane surfaces. Using robotic gridding of scFv clones on antigen-coated nitrocellulose membranes and detection by protein A/L, de Wildt et al. [24] screened over 8000 individual clones after a single selection round to identify a rare scFv that had desired cross-reactivity to both human and bovine forms of the test antigen. Focusing on cell-surface antigens, Wu *et al.* directly screened by membrane capture lift a 2×10^5 human phage-display library derived from tumor-infiltrating B-lymphocytes to identify rare, colon tumor-specific human antibodies [30]. Giovannoni *et al.* obtained high-affinity binders by initial high-density plating of a $>10^8$ library, capture onto antigen-coated membrane, detection of positive regions from the plate, and re-plating positive regions until single-colony positive isolates could be isolated [31]. A short review on membrane screening approaches can be found from Pini et al. [32].

Microarray screening

Microarray technologies have also been adapted to antibody library screening. As for protein microarrays in general [33–36], immobilization on the array surface provides a first technical hurdle. Orienting Fab fragments during immobilization, for example, through free cysteine thiol on gold, provides up to tenfold increased sensitivity compared to randomly oriented Fabs [37,38]. Site-specific biotinylation of Fabs at chain termini distant from antigen-binding sites through introduction of a biotin ligase (BirA) acceptor sequence [39,40] may also provide a means for orienting Fabs on streptavidin-coated array surfaces. In an early adaptation of immunoassays to a microarray approach, Mendoza et al. developed a capillary print head that delivered four 6×6 antigen arrays within microtiter wells and detected binding by fluorescence using a scanning CCD imager [41]. Using a streptavidin-coated array surface, onto which biotinylated antigen was coated, Pavlickova spotted scFv from periplasmic extract medium and demonstrated robust signals for typical expression culture concentrations (10–20 μg/mL), establishing the feasibility of high-throughput microarray screening using crude, *Escherichia coli*-expressed scFvs [42]. Angenendt et al. developed a multiple spotting technique (MIST) in which antigen was covalently arrayed on epoxy slides followed by spotting of crude, *E. coli*-expressed scFvs directly over antigen and detection with Cy5-labeled protein L [43]. Screening 4×96 scFv clones obtained from fourth-round phage-display selection using both enzyme-linked immunosorbent assay (ELISA) and MIST showed comparable hit-ranking. Poetz et al. proposed that relative affinity rankings of crude *E. coli*-expressed scFvs could be obtained by analyzing both antigen binding and relative scFv concentration on a single array and validated the method using a set of 62 recombinant Fab fragments with affinities ranging from 1 nM to 1.2 μM, determined by independent SPR-based affinity measurements [44].

Detection methods

Improved detection methods will enable further advances in antibody library screening. Conversion of selected antibody fragment clones to alkaline phosphatase [45–48] or GFP [49–51] fusions facilitates enzyme- or fluorescence-based detection modes, respectively. Detection amplification through rolling circle amplification, mediated by an oligonucleotide primer conjugated to secondary antibody, was shown to increase detection sensitivity more than 1000-fold compared to traditional ELISA methods [52,53] and may find promising applications in antibody array screening. By direct detection of antigen–antibody interaction by surface plasmon resonance (SPR), affinity measurements have been incorporated into the screening process, either by microarray of Fabs purified in microgram quantities onto gold and using grating-coupled SPR [54] or by high-throughput sequential injection of crude expressed Fab fragments from *E. coli* using traditional BIAcore flow cells [55]. Novel detection modes, such as microcantelever-based binding detection [56], evanescence waveguide technology [57] and magnetic acoustic resonance [58] may find future applications in high-throughput screening of antibody libraries.

ELISA-based screening

Notwithstanding the higher density formats that can be achieved through membrane-based and microarray formats, microtiter plate-based ELISA has been the most extensively reported screening method during the past decade (e.g. see [18,59–63]). After two to four selection rounds using purified protein antigen, screening on the order of 10^3 clones routinely provides hit-rates of 20–40% or greater [19,20,54,64,65]. However, this number is highly target-dependent; for example, over 8000 phage clones were screened to give a single distinct Fab sequence that recognized a sulfo-tyrosine antigen [66].

The ELISA format has been adapted to provide approximate affinity rankings during the primary screening itself. Watkins et al. showed that by using limiting amounts of anti-Fab as a capture surface, equivalent amounts of Fab fragments could be immobilized from variably expressed Fab clones, and the response obtained after addition of biotin-labeled antigen and detection with peroxidase-conjugated streptavidin was proportional to the antigen–Fab affinity [67]. Alternatively, Sidhu et al. [19] performed competition ELISA-based affinity screening by adding scFv-phage

to plates coated with antigen, with or without prior incubation with 100 nM antigen. Lower relative signals in the presence of antigen correlated with higher affinity scFvs [19].

Regardless of the physical screening format, a key challenge in rapid processing of leads from antibody phage-display libraries is information management. The following sections describe ELISA as a screening prototype and general information management considerations and challenges that are faced when screening antibody phage libraries. Literature reports describing specific software and database tools and approaches are extremely limited. Therefore, to illustrate practical solutions, a few specific examples are also provided from the authors' direct experience at Dyax Corp. using the intranet application WebPhage®. This proprietary database and software tool, not freely distributed, was developed in-house and has been used extensively for internal and contract research projects since 2002.

Screening work flow and data management

Sample IDs and tracking

A representative Ab phage HTS work flow is shown schematically in Fig. 2, which begins by plating and robotic picking of individual clones from a selection output into barcoded 96-well 'Master' plates (M-Plates). A first challenge is to define unique clone names that track each sample as they are transferred to various plates in hit-picking and rearray steps. For example, plate ID and well positions in the M-Plates (e.g. M0001-A01) can provide 'Initial Names' which are retained and associated in database tables regardless of sample transfer to new locations during rearray steps. After clone picking, individual isolates are amplified either as antibody fragments displayed on phage particles or in solution [3]. These clonal antibody forms are then screened through a target-binding primary assay, such as ELISA. As shown in Fig. 2, ELISA data can be conveniently generated by transfer of amplified antibody fragment samples from 96- to 384-well assay plates using appropriate 96-tip liquid handling devices offered by many commercial vendors.

For ELISA screening, target protein is typically immobilized in assay wells and bound Fab detected using peroxidase-conjugated anti-phage or anti-Fab secondary Abs, or using protein A/L for libraries constructed with compatible framework scaffolds [68,69]. The ELISA signal from the target is compared to that from a matched reference or control protein (background signal), and clones giving a target/background ratio above a given threshold are chosen for hit rearray. DNA sequencing is commonly the most costly part of the screening work flow; therefore, often only the rearrayed positive hits are sequenced. Sequencing data are typically generated after initial PCR-amplification of Fab clones followed by dye-terminator DNA sequencing, for example, using a 96-capillary sequence analyzer [70]. All of the experimental ELISA and sequence data from the various plates must be linked together and related to a common identifier, for example, using an Initial Name as described above.

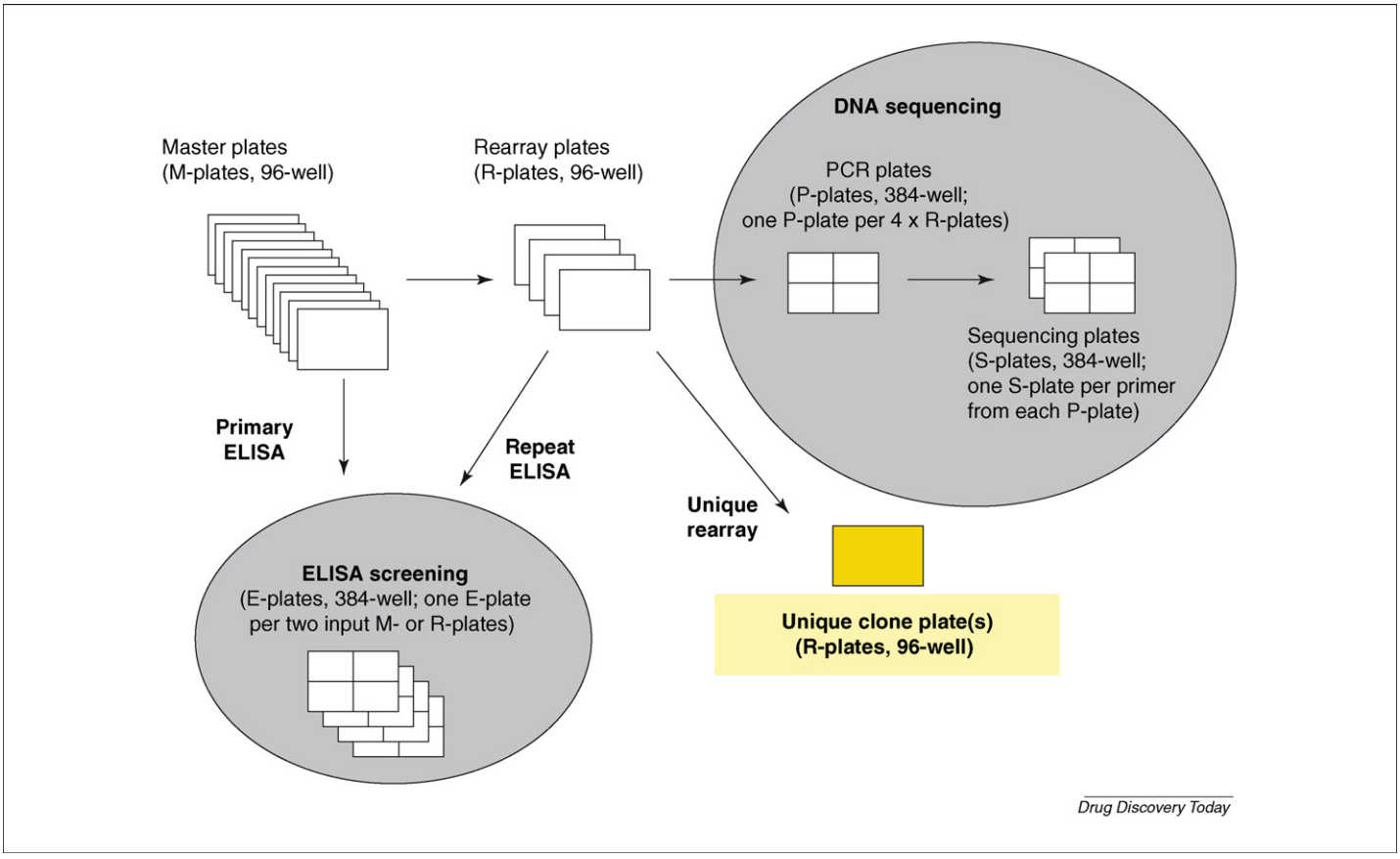

FIGURE 2

Antibody library phage screening work flow. Initial phage clones from target selection are picked into initial 'Master' plates and screened in an indirect Fab-phage or soluble Fab ELISA. Identified hits are submitted to DNA sequencing for diversity analysis. Combined analysis of assay and sequence data are used to generate a set of unique-sequence, assay-positive clones on the unique clone plates.

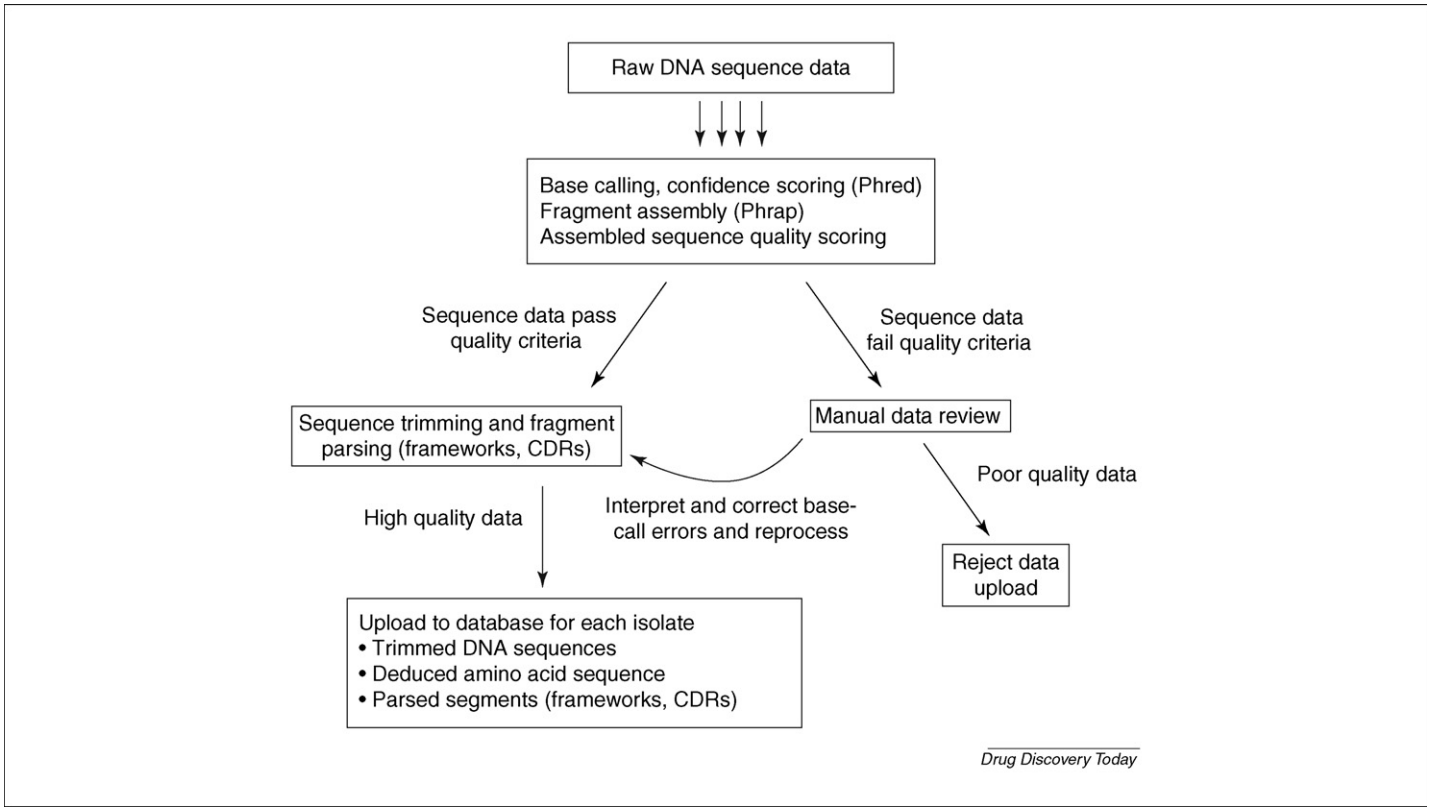

FIGURE 3

Automated processing of antibody DNA sequence data. Data from capillary DNA sequence analyzers are checked for quality, parsed into antibody sequence segments, and uploaded to a central database which allows rapid decoding of clone identity based on antibody variable region sequence.

Primary and secondary assay data management

In developing informatics systems for managing the antibody phage screening process, data generated from different assay formats or at different times must be distinguished. The following example illustrates assay data organization and viewing for a typical screening work flow. First, at the time the isolates are picked (e.g. in 96-well plates as shown in Fig. 2) it is useful to associate the samples in the database with information describing the origin of the phage pool, for example, how many selection cycles had been performed or binding and elution conditions. After reviewing the hit-rates from test screening of the various selection output samples, a subset of selection outputs can be chosen for more extensive screening, either as Fab-on-phage, as soluble Fab [54], or even after conversion to IgG [71] if the most relevant assay requires divalency. For high-throughput Fab screening, the resulting clones (e.g. 10×96 per arm) are plated, picked and amplified for use in a broad screening run. Isolates identified as target binders are rearrayed and can be processed through additional secondary assays, for example, a repeat of the screening assay to test reproducibility, or assays using variants of the target antigen to assess specificity. Isolates which produce the desired activity profile in a series of secondary assays can be further rearrayed to assess diversity by DNA sequencing.

Sequence data processing

Raw DNA sequence data from heavy and light chain of Fabs are typically processed by standard DNA sequence analysis and assembly programs such as Phred and Phrap [72] (Fig. 3). Phred assigns confidence scores for each base call and Phrap joins multiple, overlapping sequence reads into continuous sequences for each chain. Data sets not achieving defined quality scores or that lack expected sequence features can be flagged or discarded while those meeting desired criteria can be automatically uploaded to a sequence database. Flagged data sets can be manually reviewed and corrected before upload. DNA sequences can be translated into deduced amino acid sequences, trimmed at defined termini, and parsed into antibody framework and complementarity determining regions (CDRs). Regular text expression processing capabilities available through the Perl programming language [73] are especially powerful for such parsing procedures.

Parsing key diversity segments such as frameworks and CDRs facilitates further diversity analysis. For example, at Dyax Corp. using its WebPhage® intranet application, parsed segments can be sorted such that all selected clones that share the same segment sequence are grouped together and enumerated (see Table 1). During this sorting, the data are dynamically processed so that all clones sharing the same segment sequence are assigned a common 'Group Number'. The Group Number is incremented by one for each new group. By noting the final Group Number for a selected and sorted group of clones, one can readily assess the total number of clones that differ within the selected sorting segment.

Additional sequence analysis tools prove useful in further diversity and Fab sequence evaluation. For example, sequence regions

TABLE 1

Sequence group sorting[a]

Initial name	Groups	Group Reps	LV-CDR1	LV-CDR2	LV-CDR3	HV-CDR1	HV-CDR2	HV-CDR3
444L-M0144-C10	1	1	V_L (1)			V_H (1)		
444L-M0010-B11	2	2	V_L (2)			V_H (2)		
444L-M0142-G11	2	2						
444L-M0010-G03	3	2	V_L (3)			V_H (3)		
444L-M0143-F06	3	2						
444L-M0132-H07	4	4	V_L (4)			V_H (4)		
444L-M0143-D04	4	4						
444L-M0135-C01	4	4						
444L-M0132-F03	4	4						

[a] After sorting on a segment type (e.g. V_H-full amino acid or CDR3), each unique sequence group is assigned a numeric identifier (Groups column). The total repeat number for each segment sequence is tallied in Group Reps column. The maximum Group number obtained after a sorting operation is the total diversity based on the selected segment. Here, after combined V_H and V_L amino acid sorting, a total of four unique sequences, differing from other groups by at least one amino acid, were obtained for nine isolates. Further examples after sorting on different chain segments are given in Table 2B.

TABLE 2A

Representative results from selections and screening for 31 soluble proteins

Target ID #	# Screened	# Sequenced	# Unique
1	950	192	56
2	950	384	33
3	950	192	30
4	950	138	78
5	920	576	33
6	920	192	11
7	920	192	57
8	920	192	46
9	920	192	66
10	920	576	55
11	1840	192	21
12	1840	288	25
13	2760	384	66
14	864	384	96
15	1140	15	9
16	1140	181	54
17	1140	163	12
18	1140	191	40
19	1140	12	9
20	1440	90	29
21	960	104	45
22	1920	283	58
23	1920	192	21
24	960	187	41
25	864	192	14
26	1344	192	25
27	1920	288	76
28	1920	384	36
29	1920	484	254
30	1920	305	39
31	3072	158	94

that contain specific restriction sites or stop codons that would preclude defined cloning strategies or protein expression can be identified through regular expression queries, as for parsing. Selected sequences can also be compared through alignment tools or can be used as queries for BLAST searches against sequence databases, for example, for antibody germline classification or to determine similarity to all database entries.

Hit selection and rearray

Screening informatics systems must generate hit-lists for robotic rearray of initial lead candidates. For example, scalar filters can be applied to target signal (T), background signal (B) or their ratios to rapidly identify hits among thousands of screened isolates for rearray before sequencing or further specificity testing. The assay data filtering procedure typically generates a database-resident 'hit-list' that can be exported to robotic rearray devices to condense the selected leads into new set of rearray microtiter plates. After completing rearray steps, the informatics system must track the mapping between wells on source and destination plates.

Representative results from Ab phage-display HTS

To illustrate typical phage-antibody library screening results, Table 2A summarizes data from the authors' laboratory using the Dyax Fab310 library [21] and selecting and screening a set of 31 soluble protein targets using soluble Fab form (as described in [54]). From these compiled data, the overall hit-rate was 18%; on average, 36 distinct Fab clones were found per 1000 isolates screened. Table 2B shows an expanded diversity analysis for the last five sets of Fabs in Table 2A, where amino acid sequence diversity was rapidly assessed based on either combined V_H/V_L sorting or sorting on individual CDR, V_H or V_L segments. The numbers in the table represent the total number of distinct sequences obtained when sorting on the diversity segments shown in the header. As derived from data in Table 2B, on average 92% (range 66–100% for each of the five sets) of the Fabs within each target group that are unique based on full V_H/V_L do not share V_L with other members, and 91% (range 79–98%) do not share H-CDR3 with other members.

TABLE 2B

Segment diversity analysis for Fabs against five protein targets[a]

Target ID #	V_HV_L	V_H	H-CDR1	H-CDR2	H-CDR3	V_L	L-CDR1	L-CDR2	L-CDR3
27	**76**	**76**	71	71	71	74	53	33	69
28	**36**	34	32	32	32	34	22	22	28
29	**254**	252	185	243	200	167	122	90	138
30	**39**	38	38	38	37	**39**	**39**	32	**39**
31	**94**	**94**	93	91	92	**94**	36	62	82

[a] Using grouping tools (see text and Table 1), the segment diversity (shown in column headers) for the last five entries in Table 2A were determined. For example, isolates sequenced for Target #27 had 76 distinct sequences that differ by at least one amino acid in the variable region of both heavy and light chains. This same group had 71 distinct H-CDR3 but only 33 distinct L-CDR2 sequences. Segment diversity that equals full V_HV_L sorting diversity is shown in bold.

Conclusions

This overview has summarized screening methods for antibodies derived from phage-display libraries. Membrane and microarray formats allow very high screening throughput. Nonetheless, standard microtiter plate ELISA-based methods have provided a robust and convenient format that has been used most extensively in the field. Binding or activity assays and sequence data must be reviewed and cross-correlated to rapidly identify a subset of leads from thousands of clones that fulfill the desired specificity and sequence diversity requirements. These leads can then be advanced into other predictive assays to select candidates for drug development. Increasingly efficient and high-throughput screening methods together with suitable software analysis tools will continue to help research and discovery programs fill the pipeline with promising antibody leads for the foreseeable future.

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