



# Towards proteome scale antibody selections using phage display

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*In vitro* antibody generation by panning a large universal gene library with phage display was employed to generate antibodies to more than 60 different antigens. Of particular interest was a comparison of pannings on 20 different SH2 domains provided by the Structural Genomics Consortium (SGC). Streamlined methods for high throughput antibody generation developed within the ‘Antibody Factory’ of the German National Genome Research Network (NGFN) were demonstrated to minimise effort and provide a reliable and robust source for antibodies. For the SH2 domains, in two successive series of selections, 2668 clones were analysed, resulting in 347 primary hits in ELISA. Half of these hits were further analysed, and more than 90 different scFv antibodies to all antigens were identified. The validation of selected antibodies by cross-reactivity ELISA, western blot and on protein microarrays demonstrated the versatility of the *in vitro* antibody selection pipeline to generate a renewable resource of highly specific monoclonal binders in proteome scale numbers with substantially reduced effort and time.

## Introduction

A major post-genome objective is to fully characterise the human proteome. Approximately half of the open reading frames (ORFs) identified by bioinformatic analysis of the human genome encode potential protein products with unknown biological functions and, for a large fraction of proteins, function is assumed based on homology, unsupported by experimental evidence. To establish a link between sequence and phenotype, antibodies can help in many ways. Besides their classic application to identify the when, where and abundance of their antigen in immunostaining assays, they can improve proteome analysis by MALDI (Matrix

Assisted Laser Desorption/Ionisation) mass spectrometry [1]. Further, the interactions between the proteins (interactomics) prove to be the next challenge in understanding gene function. Here, antibodies promise to be broadly applicable tools to study interactions in different unmodified cells and tissues, for example, by pull down experiments [2]. To explore the full complexity and function of all these unknown ORFs and their interactions, it is essential to establish a comprehensive, characterised and standardised collection of antibodies directed against all human proteins, including variant forms and modifications [3–6]. However, only a small fraction of the proteome is covered so far, and some of the available antibodies are of questionable quality [7]. Many antibodies have been raised to work preferentially on immunoblots, that is on denatured proteins, rendering them useless for functional assays. Finally, a large number are less defined polyclonal sera with limited availability.

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Antibody phage display [8–10] allows one to select binders to almost any target, including non-immunogenic, highly homologous, toxic or allosteric variants, because the selection is based solely on binding and the biochemical milieu can be completely defined during the selection process. Thereby, it should also be possible to obtain antibodies to several interesting targets that normally would evade the immune response in experimental animals, in particular for mouse model studies. This technology has already been favourably compared to classical mouse monoclonal antibodies by raising sets of binders to the same set of cDNA derived proteins [11], showing that the affinity of respective reagents is comparable to or better than that of mouse monoclonals, if a high quality library is used. Moreover, recombinant antibody properties can be improved after selection. Miniaturisation and standardisation, in combination with primary binding analysis with soluble scFv fragments instead of antibody phage, have facilitated the multiplexed generation of scFv antibody fragments [12,13]. The use of Hyperphage allows for a reduced number of panning rounds and assures both high diversity and a high number of primary hits [14,15]. The *in vitro* antibody generation process further allows one to include selection against cross-reacting antigens during panning [16], a capability very much needed when analysing homologous protein families, and to design the antibody binding properties with regard to the needs of the biological assay. Finally, antibody phage display, because not dependent on animals, is not only ethically preferable in large-scale projects, but also amenable to automation and miniaturisation, thus significantly shortening the time from antigen delivery to antibody production and lowering the cost per binder [17]. This also conveniently fits to the growing list of miniaturised and parallelised antibody validation assays, in particular using microarray-based methods [18–20].

SH2 (Src-homology 2) domains are internal regions of a wide variety of proteins, which can contain one or several of these modules of ~100 amino acids that bind to specific phosphotyrosine-containing peptide motifs of other proteins in many different signal transduction cascades [21,22] (<http://pawsonlab.mshri.on.ca>). The typical SH2-domain fold is composed of an anti-parallel  $\beta$ -sheet sandwiched between two  $\alpha$ -helices. It forms a positively charged pocket on one side of the  $\beta$ -sheet to bind the phosphotyrosine moiety. The Structural Genomics Consortium (SGC, [23,24]) initiated a pilot project generating binders to 20 different SH2 domains [25]. Here, we describe the initial generation of these binders by phage display as an example validating this pipeline to generate quickly sets of research antibodies with considerable throughput.

## Materials and methods

### Antigens

Standard media, buffers and procedures are described in Ref. [26]. The different human SH2 domains were subcloned into the bacterial expression vector pET28 (Novagen). The resulting clone collection is commercially available through Open Biosystems, Huntsville, USA (OHS4902). The expression vectors harbouring the genes of interest were transformed to the *E. coli* strain Rosetta 2 (DE3) R3 (SGC Oxford). Inoculation cultures were grown in 10 mL Terrific Broth (TB) (Formedium) supplemented with 8 g/L glycerol, 100  $\mu$ g/mL kanamycin and 34  $\mu$ g/mL chloramphenicol at 30°C

overnight. The overnight cultures were used to inoculate large-scale cultures of 0.75 L TB supplemented with 8 g/L glycerol, 50  $\mu$ g/mL kanamycin and approximately 250  $\mu$ L Antifoam 204 (Sigma). The cultures were grown in a 'LEX' bioreactor system (Harbinger Biotechnology) at 37°C until OD<sub>600</sub> reached ~2. The cultures were cooled to 18°C over a period of one hour before target expression was induced by the addition of 0.5 mM IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside). Expression was allowed to continue overnight and cells were harvested the following morning by centrifugation (5000  $\times$  g, 10 min, 4°C). The resulting cell pellets, on average 16.9 g cells/cultivation, were resuspended in lysis buffer (50 mM NaPO<sub>4</sub>, 500 mM NaCl, 10% Glycerol, 10 mM imidazole, 0.5 mM TCEP (tris(2-carboxyethyl)phosphine), pH 8.0) (1 mL/g cell pellet), supplemented with Complete EDTA-free protease inhibitor (Roche Applied Science, 0.5 tablet/cultivation) and benzonase (Merck, 1000 U/cultivation). The cell suspensions were stored at –80°C.

The cell suspensions were quickly thawed in water. The cells were then disrupted by sonication (Vibra-Cell, Sonics) at 80% amplitude for 3 min effective time (pulsed 4 s on, 4 s off). Cell debris was removed by centrifugation (49,100  $\times$  g, 20 min, 4°C) and the supernatant was decanted and filtered through a 0.45  $\mu$ m flask filter.

Purification of the proteins was performed as a two-step process using IMAC (immobilised metal affinity chromatography) directly followed by gel SEC (size exclusion chromatography). The chromatographies were done on an ÄKTAexpress system (GE Healthcare) using 1 mL HiTrap Chelating HP columns and HiLoad Superdex 75 Prep Grade columns (GE Healthcare). Before purification, the columns were equilibrated with IMAC wash1 buffer (50 mM NaPO<sub>4</sub>, 500 mM NaCl, 10% Glycerol, 10 mM imidazole, pH 7.5) and gel filtration buffer (PBS: 10 mM NaPO<sub>4</sub>, 154 mM NaCl, pH 7.5), respectively. The filtered lysates were loaded onto the Ni-charged IMAC columns and washed with IMAC wash1 buffer followed by IMAC wash2 buffer (50 mM NaPO<sub>4</sub>, 500 mM NaCl, 10% Glycerol, 25 mM imidazole, pH 7.5). Bound protein was eluted from the IMAC column with IMAC elution buffer (50 mM NaPO<sub>4</sub>, 500 mM NaCl, 10% Glycerol, 500 mM imidazole, pH 7.5) and automatically loaded onto the gel filtration columns. Fractions containing the target proteins were pooled and protein concentrations were measured using a Nanodrop spectrophotometer (Thermo Scientific) and the resulting batches were quickly frozen in liquid nitrogen and stored at –80°C. The identities of the proteins were confirmed by mass spectrometry.

### Coupling of SH2 domains to ELISA plates and Dynabeads®

SH2 domains were immobilized on 96-well ELISA (enzyme-linked immunosorbent assay) plates (MaxiSorp™, NUNC, Wiesbaden, Germany) in 50 mM Na<sub>2</sub>CO<sub>3</sub> buffer, pH 9.2, overnight at 4°C. Following that, wells were blocked with 2% (w/v) M-PBST 0.05% in PBST (phosphate buffered saline + 0.05% Tween 20) for at least 30 min at room temperature. For the immobilisation of SH2 domains on Dynabeads® (M-270 Carboxylic Acid, Invitrogen Dynal, Oslo, Norway) 10 mM sodium acetate buffer, pH 5.0, was used. The carboxylic surface of 1–2  $\times$  10<sup>7</sup> beads activated before immobilisation with a 1:1 mixture of 0.1 M NHS (N-hydroxysuccinimide and 0.4 M EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide); both GE Healthcare) for 8 min at room

temperature using an overhead rotator. 0.5–2 µg of SH2 proteins were coupled in acetate buffer for 20 min at room temperature. After immobilisation, beads were saturated using 50 mM ethanolamine in PBS and blocked with 1% (w/v) BSA in PBS 0.1% Tween 20.

#### Selection of recombinant scFvs

The panning procedure was based on published protocols [27] with modifications, in 96-well ELISA plates (Maxisorb™, NUNC, Wiesbaden, Germany) and on paramagnetic beads. SH2 domains were immobilised as described above, followed by blocking with 2% (w/v) skimmed milk in PBS 0.1% Tween 20 (2% M–PBST 0.1%) for at least 30 min at room temperature. Wells were washed three times with PBST using TECAN ColumbusPro ELISA washer (Tecan Austria GmbH, Grödig, Austria).

The human naïve HAL4 and HAL7 antibody gene libraries [27–29], based on the phagemid vector pHAL14 [30] and consisting of  $>5 \times 10^9$  independent clones, were used for panning. The libraries were initially packaged using Hyperphage [14]. During the first round of pannings for each SH2 domain, four wells coated with the respective SH2 domain were used. Before panning  $2.5 \times 10^{11}$  scFv phage particles (cfu) of HAL4 (Vκ repertoire) and  $2.5 \times 10^{11}$  scFv phage particles (cfu) of HAL7 (Vλ repertoire) were diluted in 150 µL 1% M–PBST 0.05% per well. In a preselection step, the library phage suspension was incubated at room temperature for 30 min in wells blocked with 2% M–PBST 0.1% before being transferred to the wells with the immobilised SH2 domains. After an incubation for 1.5 hours at room temperature and 300 rpm in a microtitre plate shaker (Thermoshaker PST60-HL4, Lab4You, Berlin, Germany), wells were washed 20 times with PBST. Bound scFv phage particles were eluted with 170 µL trypsin solution (10 µg/mL trypsin in PBS) at 37°C for 30 min. The supernatant containing the eluted scFv phage was used to infect 10 mL of an exponentially growing culture of *E. coli* XL1-blue MRF' (Stratagene, Amsterdam, Netherlands) at OD<sub>600nm</sub> ~0.6 and incubated for 30 min at 37°C and 110 rpm. Dilutions were plated on 2xYT GAT (2xYT containing 100 µg/mL Ampicillin, 100 mM glucose and 20 mg/L tetracycline) agar plates for phage titration. Remaining culture was harvested by centrifugation for 5 min at 3200 × g. The pellet was dissolved in 250 µL 2xYT GA and spread onto 2xYT GAT agar plates. Plates were incubated overnight at 37°C and phage amplification was performed as described [27] and used for the next panning round. The two subsequent additional rounds of pannings were performed using M13KO7 [31] as helper phage and with the following protocol modifications: (i) incubation buffer I (1% (w/v) M–PBST 0.05%) and incubation buffer II (1% (w/v) BSA (bovine serum albumin) in PBS 0.05% Tween 20) were alternated in successive panning rounds, (ii) the antigen amount was reduced by 50% compared to the previous round each time, (iii) washing cycles were increased to 40 times per panning round, and (iv) phage input was reduced to approximately  $1 \times 10^{10}$  cfu in total.

Panning conditions and procedure on paramagnetic beads (Dynabeads® M-270 Carboxylic Acid, Invitrogen Dynal, Oslo, Norway) were identical to those of the ELISA plate pannings. Bead numbers were reduced to 50% after the first round of pannings, the target amount was reduced by half in each panning round, washing steps were doubled and phage input decreased by 50% from round to round.

#### Production of soluble antibody fragments

Soluble scFvs were produced in microtitre plates [13]. Individual clones were randomly picked both after the second and third panning round to inoculate master microtitre plates (150 µL 2xYT GA per well) and incubated overnight at 37°C with constant shaking at 850 rpm. 200 µL fresh 2xYT GA was inoculated with 10 µL of the overnight culture and grown at 37°C and 850 rpm for two hours. After centrifugation for 10 min at 3200 × g at 4°C, the pellets were resuspended in 200 µL 2xYT + 100 µg/mL ampicillin + 50 µM IPTG, and incubated at 30°C and 850 rpm overnight. After centrifugation for 10 min at 3200 × g and 4°C, the scFv were collected with the supernatant.

#### ELISA

Supernatants were analysed by antigen ELISA on SH2 domains immobilised on Nunc Maxisorb™ ELISA plates or paramagnetic beads (100 ng SH2 per ELSA plate well, 10 ng SH2 per 1 µL paramagnetic beads per microtitre plate well). Wells and beads were blocked with 2% M–PBST 0.05%. 50 µL of the supernatant from soluble scFvs produced microtitre plates were incubated in 1% M–PBST 0.05% final concentration in a total volume of 100 µL per well for one hour at room temperature, followed by three washes with PBST. Bound soluble antibody fragments were detected by using the murine anti-c-myc mAb Myc1-9E10 [32]. Staining was performed with a goat anti-mouse Ab conjugated to horseradish peroxidase (HRP) (Sigma; 1:10,000). After staining with 3,3',5,5'-tetramethylbenzidine (TMB, Sigma), the reaction was stopped with 1 M sulphuric acid. Absorbance at 450 nm was measured by SUNRISE™ plate reader (Tecan, Crailsheim, Germany). Selected different scFvs were further tested in ELISA on lysozyme and all SH2 domains as control targets using the same protocol.

#### DNA analysis

347 individual scFv clones binding their respective SH2 antigen in ELISA were subjected to BstNI/AluI fingerprinting (restriction endonuclease digestion followed by agarose gel electrophoresis) to classify clones with identical restriction patterns (data not shown). About half of the clones identified to be different in the BstNI/AluI fingerprinting were further subjected to DNA sequencing of the scFv insert (GATC, Konstanz, Germany).

#### Immunoblotting

1 µg of each SH2 domain was blotted from a 15% SDS-polyacryl gel electrophoresis (SDS-PAGE) onto a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 1% M–PBST 0.05%. ScFv dilutions in PBST were incubated for one hour and detected by Myc1-9E10 followed by anti-mouse Fc-Ab conjugated to alkaline phosphatase. Binding was visualised with 5-bromo-4-chloro-3-indolylphosphate (BCIP)/nitro blue tetrazolium chloride (NBT) substrate (Sigma, Germany).

#### Binding of scFv fragments on antigen microarrays

Microarray assays were generated and analysed as described [19]. The microarrays were spotted onto epoxy-coated slides (Corning Life Sciences) with 14 identical subarrays on each slide utilising a non-contact arrayer, Nanoplotter 2.0E (GeSim). Each subarray contains 432 polypeptides namely: 301 PreSTs (protein epitope signature tags) encoded on human chromosome 21, 85 PreSTs

corresponding to 53 unique SH2-domain containing proteins and duplicates and triplicates of the 20 folded SH2 domains. The latter group was spotted at different concentrations ranging from 0.4 to 4.0 mg/mL and also 1:1 dilutions of each. The PRESTs were diluted to 40 µg/mL in 0.1 M urea and 50 mM sodium carbonate–bicarbonate buffer, pH 9.6, complemented with 100 µg/mL BSA.

Slides were blocked in 3% BSA in PBST before incubation. The scFvs were incubated at dilutions ranging from 0 to 1:50 in PBS for one hour. The secondary antibody (mouse hybridoma supernatant, anti-cmyc Myc1-9E10 [32]) was incubated at 1:1000 dilution in 1% PBS for one hour and the tertiary antibody (goat anti-mouse-Alexa 647, Molecular Probes, Invitrogen) was used at 1:1000 dilution in PBS. The slides were scanned (G2565BA array scanner, Agilent) and images quantified using image analysis software (GenePix 5.1, Molecular Devices).

## Results

### Antibody selection

Generating recombinant antibodies by *in vitro* selection was achieved rapidly when compared to animal-based methods, as illustrated by the project timeline (Fig. 1). Before selections were started, the most efficient way for antigen immobilisation to microtitre plates was sought. For some target proteins (LCK, CRK, BCAR3, PIK3RI, RASA1C, ZAP70, BTK) coating in carbonate buffer instead of PBS produced better results (Fig. 2). No advantage was observed for any of the 20 SH2 domains when using PBS. As efficient immobilisation is essential for an efficient selection, care has to be taken at this very first step of Ab isolation. After three rounds of selections on each of the 20 targets, monoclonal screening was carried out using 92 clones for each SH2 domain by antigen ELISA of supernatants containing soluble scFv after overnight production in microtitre plates (Figs 3,5A). Results of this first approach varied between zero binders in the case of FYN and GRAP2 and 40 screening positives in the case of CRK (Table 1). It

should be noted that FYN and GRAP2 were among the antigens with limited solubility (Table 1).

The two antigens which yielded no scFv in the first campaign and four additional SH2 domains which yielded few or weakly reacting clones (VAV1, ZAP70, GRAP2, BTK) were subjected to covalent coupling to the carboxylic surface of paramagnetic bead particles. Optimal immobilisation was reached with 20 min of incubation under mildly acidic conditions with the EDC/NHS-activated bead surface. When compared to immobilisation on MTPs, it became evident that coupling was much more efficient on beads, and with low variation between different antigens (Fig. 4). As expected, the two different methods of antigen presentation during panning resulted in different panning results. For the two antigens where no primary hits were found in the first selection on MTPs, bead-based panning led to hits, while for the other four antigens there was a slightly better yield of both primary hits and number of different antibody clones (Table 2, Fig. 5). For the strongly precipitating antigen FYN, all three different antibodies obtained from 92 analysed clones after selection on beads showed cross-reactivity with other SH2 domains, but not with lysozyme.

### Binding to denatured antigen

After subjecting the various SH2 domains to non-reducing SDS-PAGE and immunoblotting, the proteins were probed with one respective scFv fragment per antigen to obtain an impression of how denatured domains are recognised (Fig. 6). Of 18 different scFv tested, 15 showed binding to their denatured target. Only LCK\_DM1, ZAP70\_DM1 and RASA1\_DM2 did not recognise their denatured antigens. Some reactivity was observed to bands larger than 25 kDa for LYN-DM1, RASA1-DM1, ABL2-DM1 and PLCG1-DM1 (corresponding to bands present in the purified antigen, as seen on the silver-stained gels shown in Figs. 2,4). There was no clear correlation of scFv binding on western blots to that on bead-based binding assays using the panning beads, but the six scFv

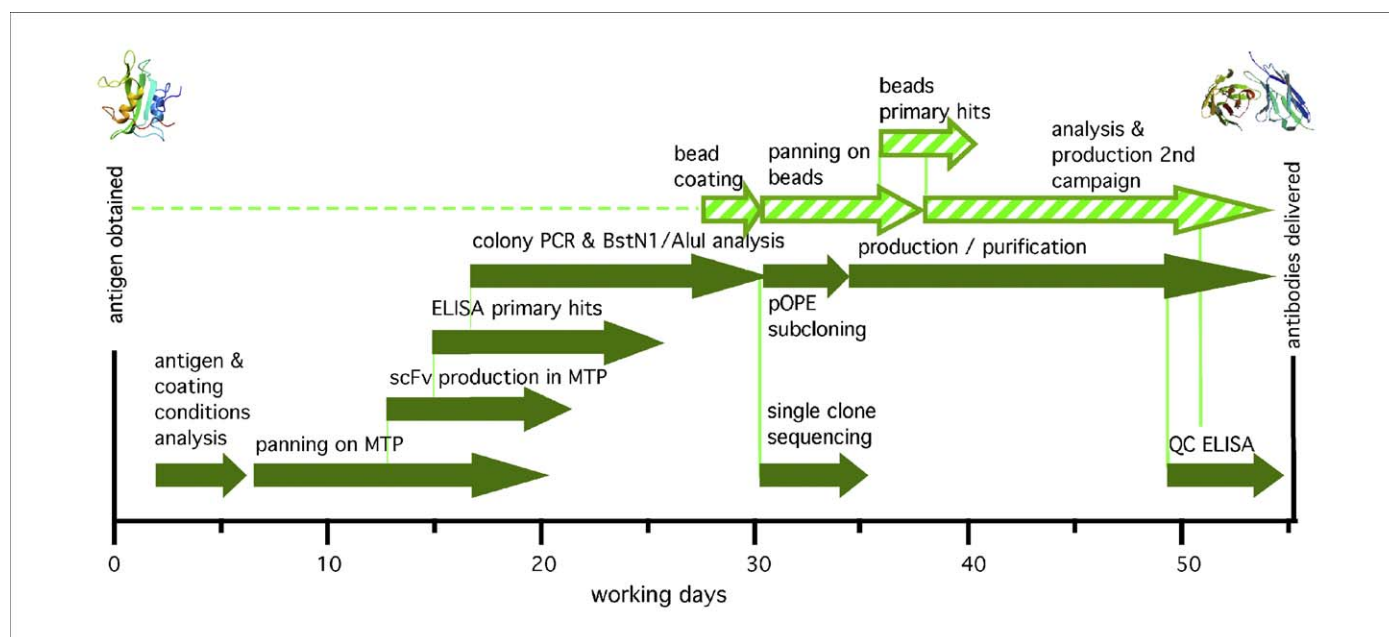


**FIGURE 1**

*In vitro* antibody generation pipeline of the SH2 project: tasks and experienced time requirements.

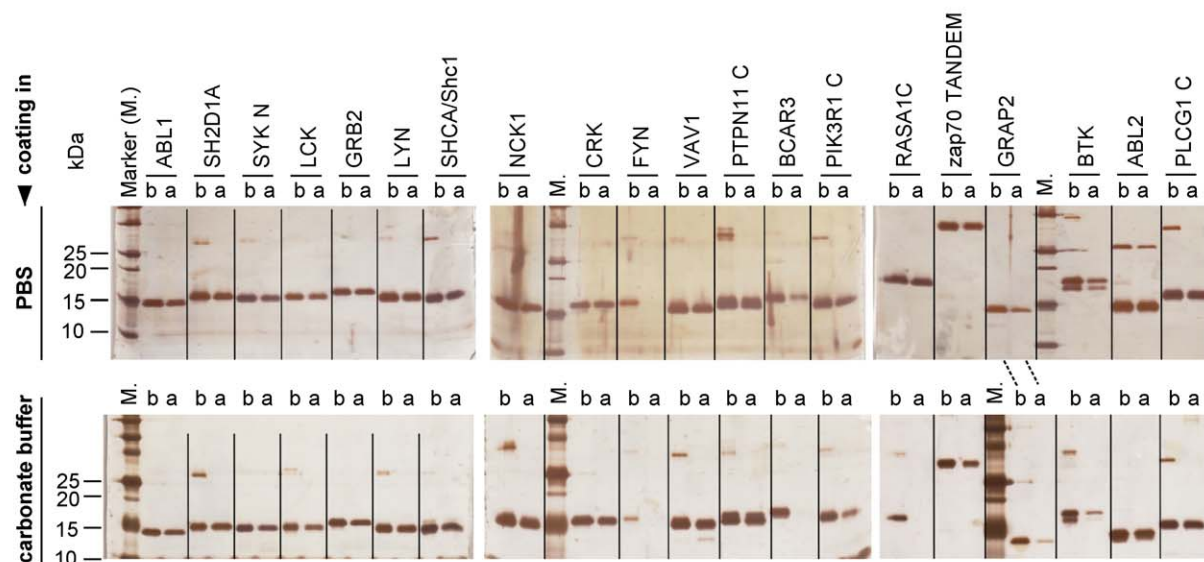


FIGURE 2

Antigen quality analysis and comparison of coating conditions. 100  $\mu$ L containing 500 ng of each SH2-domain preparation was incubated per microtitre plate well. Coating was in PBS (upper gels) and carbonate buffer (lower gels) overnight at 4°C. Per lane, 7.5  $\mu$ L samples of coating solution collected either before (lanes labelled 'b') or after (lanes labelled 'a') the coating were loaded and silver stained after SDS-PAGE. Dashed lines indicate the corresponding lanes of GRAP2.

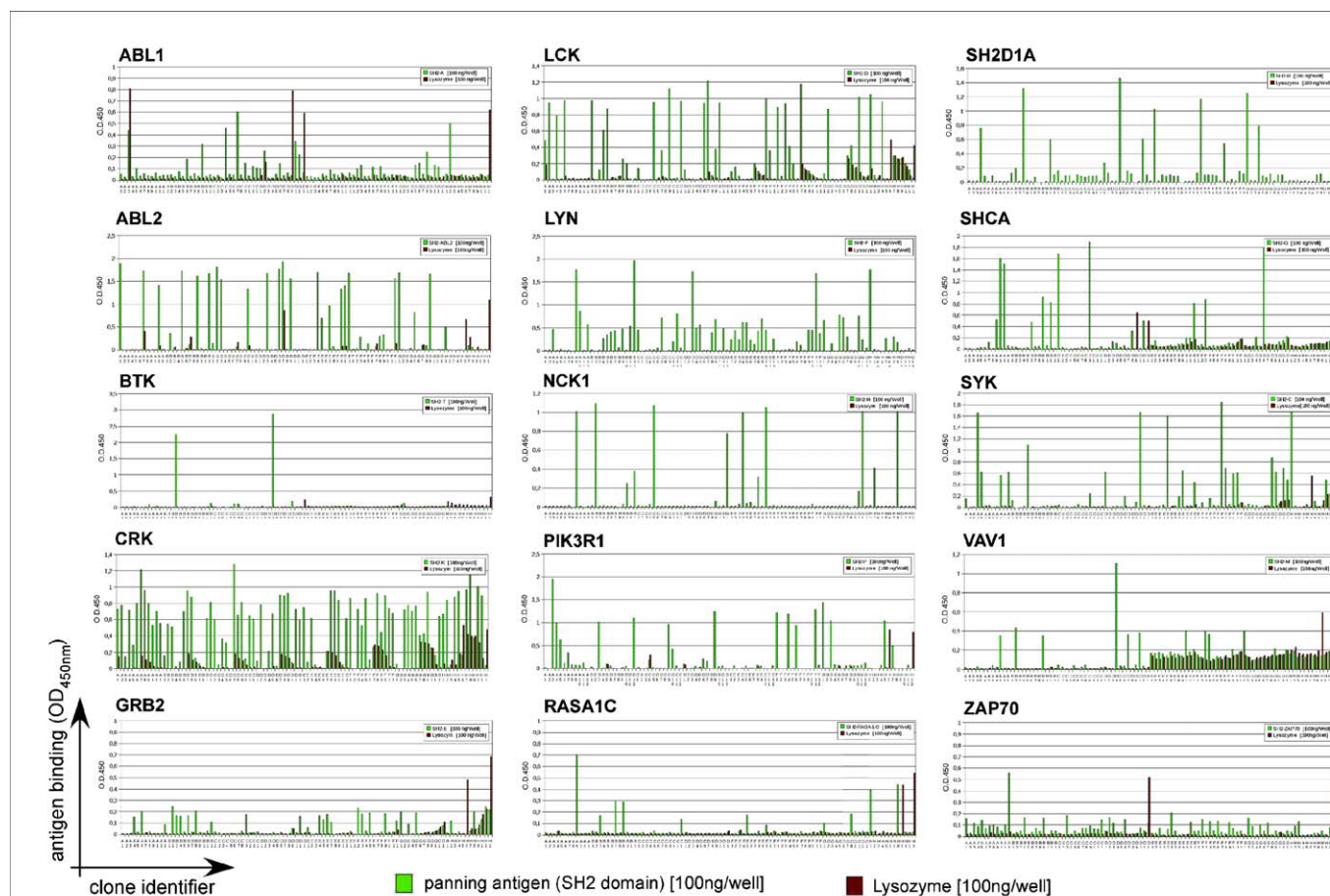


FIGURE 3

Single clone antigen ELISA data of the first screening campaign on microtitre plates. 92–96 antibody clones were randomly picked. Bars showing the binding to the panning antigen are in green, to negative control antigen (hen egg lysozyme) in brown. Respective ELISA data for GRAP2 are shown in Fig. 5.

TABLE 1

## Antigens and panning results

| Target identifier | Accession # | Antigen quality observation | Panning on MTP <sup>a</sup> |                     | Panning on beads <sup>a</sup> |                     | Origin of SH2 domain (protein name)                                                               |
|-------------------|-------------|-----------------------------|-----------------------------|---------------------|-------------------------------|---------------------|---------------------------------------------------------------------------------------------------|
|                   |             |                             | Positive in screening       | Different sequences | Positive in screening         | Different sequences |                                                                                                   |
| ABL1              | BC059260    | Precipitate                 | 5                           | 4                   |                               |                     | Proto-oncogene tyrosine-protein kinase ABL1 (Abelson murine leukaemia viral oncogene homologue 1) |
| ABL2              | BC065912    |                             | 22                          | 4                   |                               |                     | Tyrosine-protein kinase ABL2 (Abelson murine leukaemia viral oncogene homologue 2)                |
| BCAR3             | BC039895    |                             | 12                          | 3                   |                               |                     | Breast cancer anti-oestrogen resistance protein 3                                                 |
| BTK               | AY335749    | Precipitate                 | 2                           | 2                   | 68                            | 2                   | Tyrosine-protein kinase BTK (EC 2.7.10.2) (Bruton tyrosine kinase)                                |
| CRK               | BC008506    |                             | 40                          | 2                   |                               |                     | Proto-oncogene C-crk (p38)                                                                        |
| FYN               | LSEQ1749330 | Strong precip.              | 0                           | 0                   | 8                             | 3                   | Proto-oncogene tyrosine-protein kinase Fyn                                                        |
| GRAP2             | BC025692    | Foggy                       | 0                           | 0                   | 44                            | 2                   | GRB2-related adapter protein 2 (GADS protein)                                                     |
| GRB2              | BC000631    |                             | 2                           | n.d.                | 5                             | 5                   | Growth factor receptor-bound protein 2 (Adapter protein GRB2)                                     |
| LCK               | BC013200    |                             | 20                          | 3                   |                               |                     | Proto-oncogene tyrosine-protein kinase LCK                                                        |
| LYN               | BC031547    |                             | 20                          | 4                   |                               |                     | Tyrosine-protein kinase Lyn                                                                       |
| NCK1              | BC006403    |                             | 9                           | 3                   |                               |                     | Cytoplasmic protein NCK1 (NCK adaptor protein 1)                                                  |
| PIK3R1 C          | BC030815    |                             | 14                          | 5                   |                               |                     | Phosphatidylinositol 3-kinase regulatory subunit alpha (PI3-kinase p85 subunit alpha)             |
| PLCG1C            | BC065091    |                             | 10                          | 7                   |                               |                     | 1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma-1                                 |
| PTPN11 C          | BC008692    |                             | 6                           | 6                   |                               |                     | Tyrosine-protein phosphatase non-receptor type 11                                                 |
| RASA1C            | BC033015    | Foggy                       | 4                           | 3                   |                               |                     | Ras GTPase-activating protein 1 (GTPase-activating protein)                                       |
| SH2D1A            | BC020732    |                             | 10                          | 6                   |                               |                     | SH2-domain containing protein 1A (Signalling lymphocytic activation molecule-associated protein). |
| SHCA/Shc1         | BC014158    |                             | 12                          | 10                  |                               |                     | SHC-transforming protein 1 (SH2-domain protein C1)                                                |
| SYKN              | BC011399    |                             | 15                          | 4                   |                               |                     | Tyrosine-protein kinase SYK (Spleen tyrosine kinase)                                              |
| VAV1              | BC013361    |                             | 9                           | 2                   | 20                            | 8                   | Proto-oncogene vav.                                                                               |
| zap70 TANDEM      | LSEQ3295505 |                             | 2                           | 1                   | 3                             | 2                   | Tyrosine-protein kinase ZAP-70                                                                    |

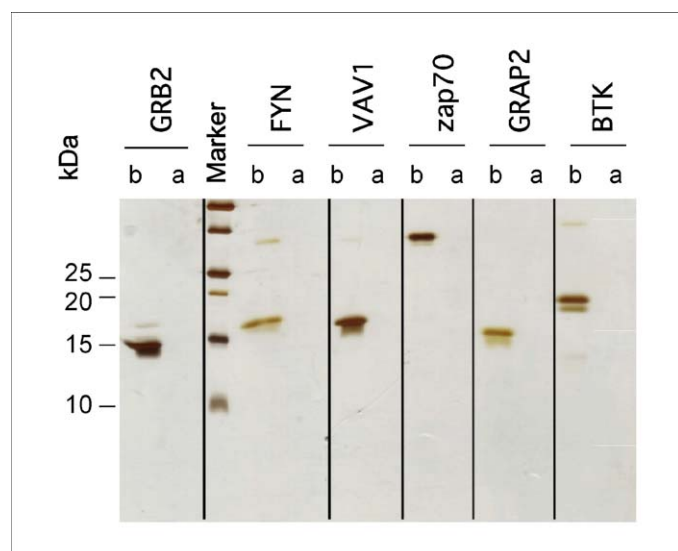
<sup>a</sup> For all targets and per method, 92 individual clones were picked, soluble scFv fragments were produced in MTPs and analysed for binding to target and lysozyme (exception: BTK, FYN and GRAP2 on MTPs: 184 analysed). Positive hits did not react with lysozyme and were at least 5× over background.

antibodies reacting on beads all bound in ELISA, but not vice versa (Fig. 6). This illustrates that accessible epitopes with the conformation required for binding by the scFv can differ between ELISA plates, beads and western blots, which cannot be explained by a simple denatured/native dichotomy. The results underline the necessity to select the antibody under conditions as close as possible to the assay it is needed for.

#### Analysis of specificity and cross-reactivity

Further ELISA experiments were carried out to analyse cross-reactivities of individual scFv antibodies. In general, each scFv fragment exhibited good specificity without significant binding to the 16 other SH2 domains tested for in this assay (Fig. 7). For some primary hits, however, the assay revealed a broad cross-reactivity among all antigens even though no binding was observed to the negative control protein used in primary screening (BSA), indicating a reactivity with shared sequences. It is improbable that this common sequence is the his-tag of the antigens, because the scFv fragments also contain a very similar his-tag and thus would compete for the binding or aggregate.

For a broader analysis of nonspecific and cross-reactivities to be expected from our simplified panning pipeline, a set of antibodies not preselected for low cross-reactivity was analysed on protein arrays. In particular, one positive scFv clone per SH2 antigen was selected and further analysed to evaluate the primary quality of binders. The arrays contained 301 PreSTs from various protein classes, including 53 SH2-domain proteins, plus the 20 folded SH2 domains used for panning in duplicates/triplicates. A substantial number of scFv provided specific detection of their target SH2 domains (typical pattern shown in Fig. 8A,H). Significantly, cross-reactivities with the 386 spots of denatured proteins were surprisingly rare (Fig. 8A,B,E–H). This strongly indicates that the scFv fragments generally were of good quality, with a very low fraction of unfolded/denatured and therefore sticky proteins. Interestingly, in most cases, reactivity with the PreSTs corresponding to the folded SH2 domain was not observed, despite sometimes showing reactivity in immunoblots of the panning antigen. In one case, a defined cross-reactivity to a single non-related PreST was detected (Fig. 8D). Other scFv fragments showed cross-reactivity with some other folded SH2 domains (Fig. 8F). An anti-ABL2 scFv fragment

**FIGURE 4**

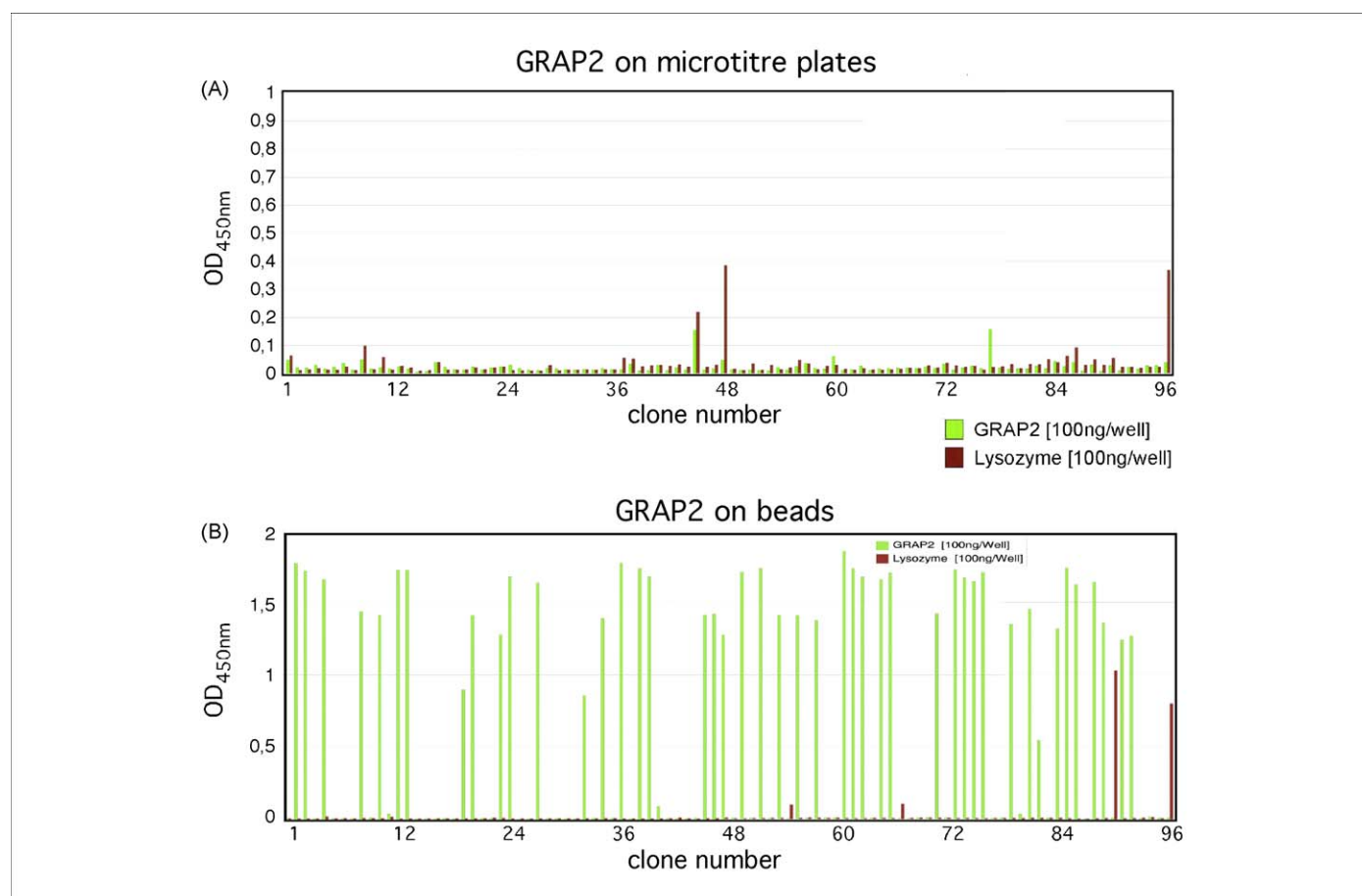
Covalent coupling of paramagnetic beads to selected SH2 domains. Sample collection and analysis as described in Fig. 2. Lanes: b (before), antigen solution before coupling, a (after), bead supernatant after coupling (equal fraction of total applied).

**TABLE 2****Summary of panning results**

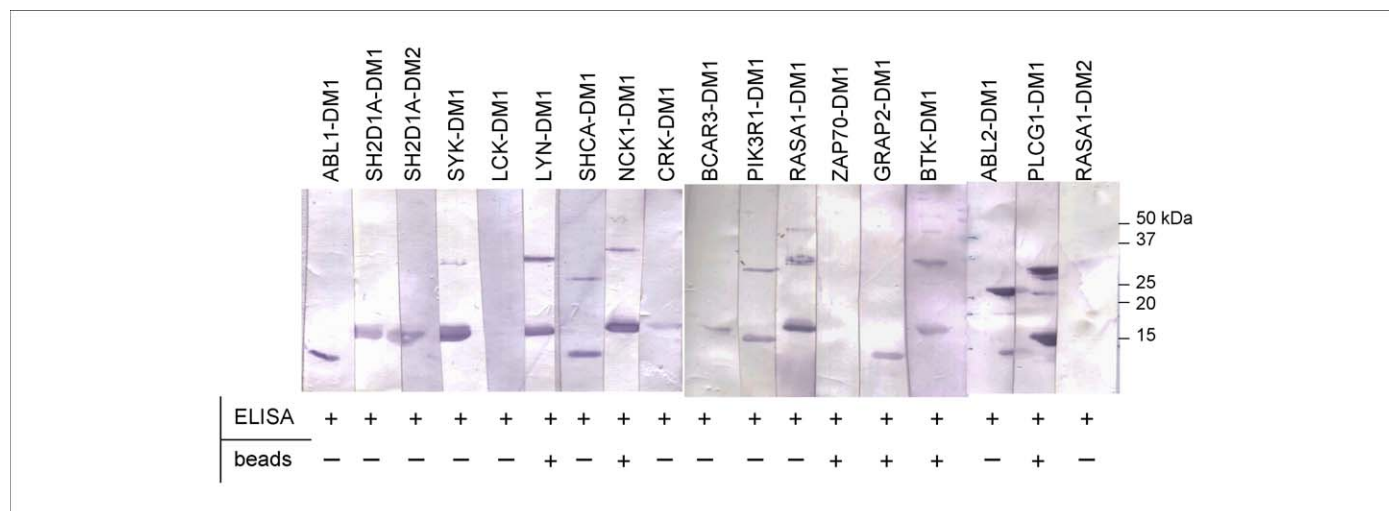
|                                                                                                   |                  |
|---------------------------------------------------------------------------------------------------|------------------|
| Targets (antigens): SH2 domains                                                                   | 20               |
| Total number of analysed clones                                                                   | 2668             |
| Primary hits MTP panning (ELISA)                                                                  | 213              |
| Number of antigens rescreened on beads:                                                           | 6                |
| Hits MTP + bead panning                                                                           | 347              |
| Average % of hits in randomly picked clones from two panning rounds                               | 13%              |
| Confirmed different antibody clones (BstEI/AluI and sequencing)                                   | 90 <sup>a</sup>  |
| Average number of different binders found per antigen in 100 randomly picked clones after panning | 4.9 <sup>a</sup> |

<sup>a</sup> Only about half of the positive hits were analysed by sequencing, so the real numbers are expected to be higher.

was strongly cross-reactive with ABL1 (Fig. 8B), which can be explained by the fact that the antigens differ by only a few amino acid positions and the panning was not performed with the addition of ABL1 antigen as a competitor. However, the ABL1 antibody analysed in cross-reactivity ELISA (Fig. 7 upper left panel) did not show strong cross-reactivity, indicating the capacity of the HAL4/7 library to provide sufficient fine specificity if panning and selection conditions are carefully adjusted. An scFv to NCK1 reacted with SYK, an SH2 domain of little homology (Fig. 8G). Two additional patterns were observed. First, scFv antibodies

**FIGURE 5**

Comparison of panning on polystyrene microtitre plates or magnetic beads. Single clone ELISA results on GRP2 or hen egg lysozyme of 92 randomly picked clones after panning on microtitre plate-immobilised GRP2 (A) or bead-immobilised GRP2 (B).

**FIGURE 6**

Immunoblots with scFv fragments. 1 µg of each SH2 domain was run on a 15% SDS-PAGE gel. ScFv fragments were used to stain immunoblots of their corresponding antigens. Detection: anti-myc-tag-antibody followed by an anti-mouse AP conjugate. Staining with NBT + BCIP. The reactivities of the analysed scFv clones to the same antigen in ELISA and on bead-based binding assays are indicated below the blot.

which detected their specific antigen well and without cross-reactivity among the folded SH2 domains, but with a generally high nonspecific binding to all denatured proteins (Fig. 8C). Conversely, pan-SH2 antibodies were detected (Fig. 8E) which reacted with all folded SH2 domains, but with no denatured proteins. In the first case, anti-tag reactivity might be a possibility, even though during the panning no PrEST was included in the antibody selection chain, arguing against this explanation. It has to be mentioned that scFv fragments were used here on this array for the first time. Without any optimisation of the detection protocol, many of them performed well on the array and despite their monovalent nature, provided sufficient affinity and signal to noise ratios.

## Discussion

In two successive selection campaigns over two months, 2668 individual antibody clones to 20 different SH2 antigens were analysed. From 347 primary hits in ELISA, with only half of the hits characterised further, 90 different scFv fragments against all 20 SH2 domains were identified by BstNI/AluI fingerprinting, of which 45 were verified by DNA sequencing. Monoclonal scFv from that pool generally performed well in ELISA, microarrays and in western blotting, despite folded antigen having been used for the selection, suggesting that a mixture of linear and conformational epitopes are recognised by the selected binders. Despite the finding that some of the initial hits did not perform satisfactorily in some of the antigen binding assays, the pipeline promises excellent success rates for future high throughput selection of antibodies, as the analysis of more clones or rescreening with different targets corrected these deficiencies in a short time, as shown for GRAP2 and ABL1. Despite the limited number of samples analysed and the high homology within the antigen group, panning on beads with covalently coupled antigen yielded better overall success rates. In some instances, the functionality of the isolated scFv fragments correlated with the applied panning method. This has been described in an even more pronounced fashion [28], where a prevalence of conformational epitopes was found for binders from

bead-based pannings, whereas ELISA-well panning led to a strong prevalence of sequential epitopes. This emphasises the necessity to run the selections under conditions as close as possible to the final assay the scFv fragment will be used in.

In respect of process optimisation, this pilot project has revealed important parameters of antigen coating and panning procedures. With averages of 13% positive primary hits and more than four different monoclonal scFv per 92 analysed eluted clones, this setup of the pipeline can be expected to yield binders while using only a single microtitre plate per antigen for initial screening. The HAL4/7 library and the process pipeline were further validated in other projects by raising antibodies to more than 40 other antigens, including some where other antibody generation techniques have failed or have been found to be difficult. Antibody fragments to haptens, synthetic peptides, a diverse sets of other proteins and pathogens including whole viruses [28,29], and interestingly, even to *E. coli* LPS could be raised.

However, the results emphasise the necessity for more sophisticated primary specificity assays. Clearly, clones with broad cross-reactivity within a homologous protein family can evade exclusion in the primary screening ELISA when simple negative controls, like BSA or lysozyme, are employed, as in most current primary assays. Here, protein arrays [18–20] will prove to be valuable in the future, because they allow one to assess binding to a large number of proteins using only minute amounts of scFvs.

The comparison of scFv function between ELISA, western blots, bead-based binding assays and microarray data emphasised again the necessity to generate different binders appropriate for different assays. Here, the *in vitro* panning step inherent to phage display offers to enhance the chances of finding an antibody working for a particular assay, because the biochemical condition, antigen quality and epitope optimal for that assay can easily be adjusted in the panning reaction, even enabling selection of binders specific for a single distinct allosteric conformation of a protein [33]. If available, the use of homologous proteins as soluble competitors during panning should be employed to minimise the isolation of cross-reacting binders from the start.

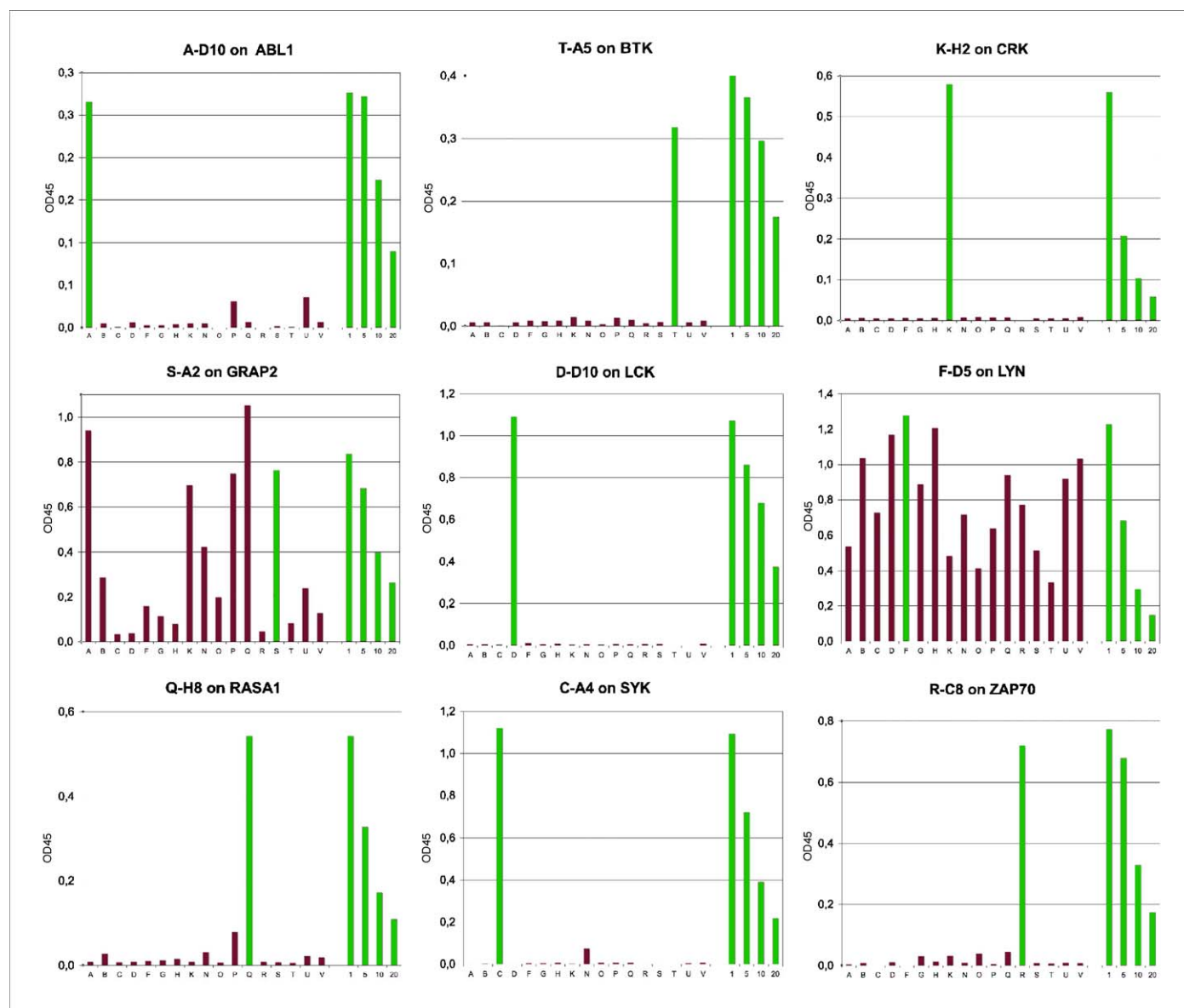


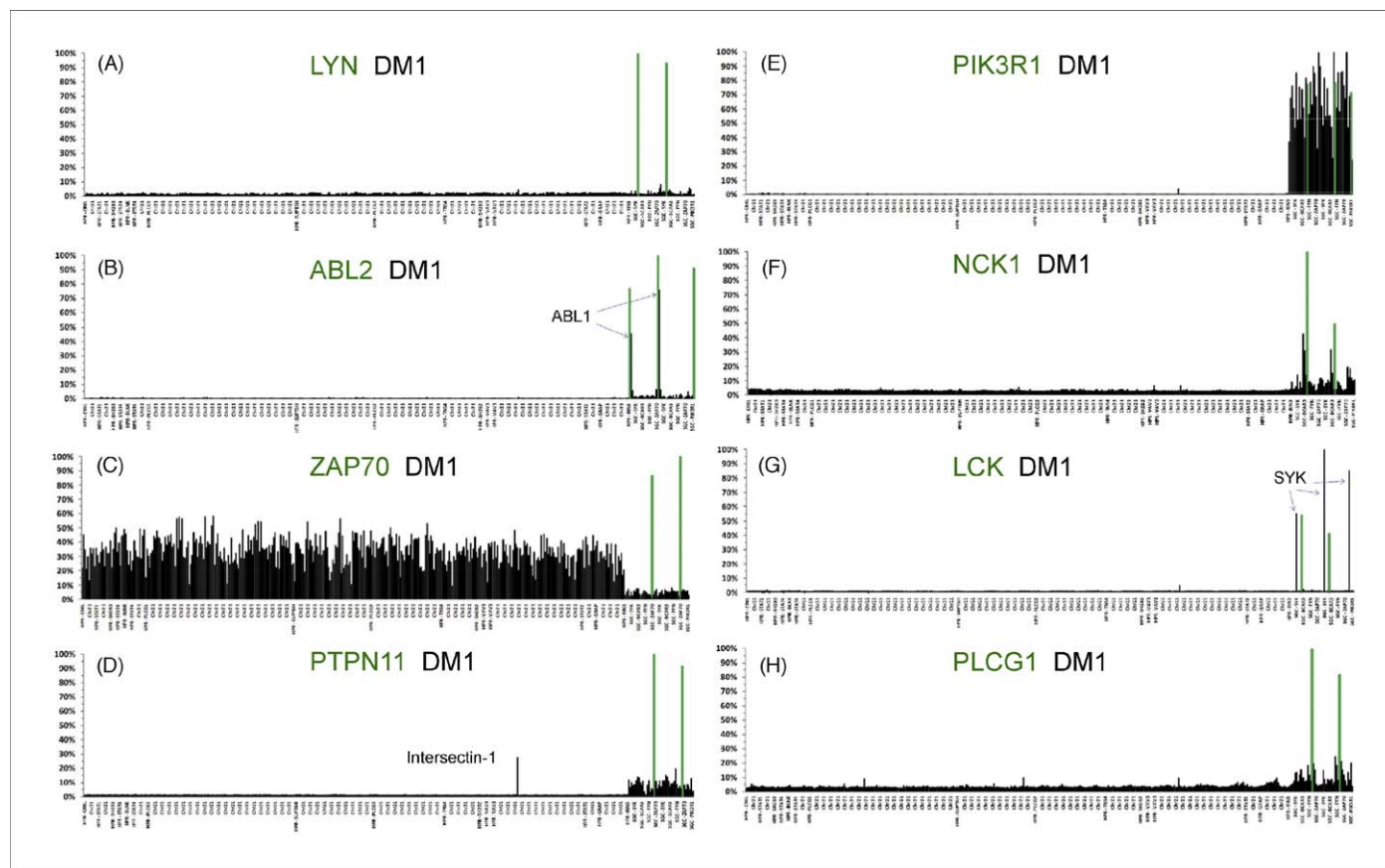
FIGURE 7

Cross-reactivity ELISA using *E. coli* culture supernatants on the soluble antigens. Targets are abbreviated as follows: A: ABL1, B: SH2D1A, C: SYK, D: LCK, E: GRB2, F: LYN, G: SHCA, H: NCK1, K: CRK, L: FYN, M: VAV1, N: PTPN11, O: BCAR3, P: PIK3R1, Q: RASA1, R: ZAP70, S: GRAP2, T: BTK, U: ABL2, V: PLCG1. The bar showing the reactivity with the panning antigen is in green. The four rightmost columns indicate reactivity to the corresponding panning antigen in scFv supernatant dilutions of 1:1, 1:5, 1:10 and 1:20 (left to right).

Average monovalent affinities of primary hits from universal antibody libraries are usually distributed in the double-digit nanomolar range, matching the monovalent affinities of animal-derived monoclonal antibodies. From the HAL4/7 library, several scFv fragments with monovalent dissociation constants down to 3 nM have been isolated. Nevertheless, in several widely used assays, such as ELISA, immunoblotting, immunohistochemistry or FACS, conventional monoclonal antibodies and antisera benefit from the avidity provided by the bivalent nature of a full IgG. ScFv fragments can easily be adapted to be bivalent or even multivalent, for example, by fusion to *E. coli* alkaline phosphatase [12,34], streptavidin [35], biotin/streptavidin [36], calmodulin [37], leucine zippers [38], amphipathic helices [39], p53 [40], S-protein/S-peptide [41] or minibodies [42]. A recent systematic comparison identified *in vivo* biotinylation as the most efficient way to achieve

an avidity advantage with scFv fragments [43]. Antibodies matching the functionality of an IgG molecule can be obtained by single step cloning to create a scFv-Fc fusion protein [44–46].

Creating antibodies for proteome research *in vitro* using phage display adds opportunities offered by the fact that the gene encoding the binder is available. Recently, the recombinant production of scFv fragments fused to Fc portions from different species was shown to allow the simultaneous detection of three different antigens in the same cell with three different fluorescence colours, despite all antibodies originating from the same phage display library [47]. Various scFv genes have been used for the functional analysis of the target gene by expressing them inside a mammalian cell to knock down the target protein function (for review see [48]). After a single step subcloning into a mammalian expression vector, scFv genes induced a functional knockdown of the target

**FIGURE 8**

Microarray binding analysis on 374 different proteins arrayed in 432 spots, including 85 PRESTs corresponding to 53 unique SH2-domain containing proteins and duplicates or triplicates of the 20 folded SH2 domains used for panning (the latter located at the right hand end of the graph). Antibody examples were selected to represent the different observed binding patterns. In black: signals on control proteins; in green: signals on panning antigen. Signal height was set to 100% corresponding to the maximal signal of each analysed scFv antibody.

antigen, as demonstrated for receptors on immune and nerve cells, both in cell culture [49] and living primary tissue (unpublished). Owing to their human origin, scFv fragments could go straight into therapeutic development as lead compounds, for example by reconstituting a full IgG, by fusion to another binder to generate a bispecific antibody (for review see [50]) or by fusion of an effector able to kill tumour cells [46].

In conclusion, the SH2 pilot project results underline the conclusion of earlier studies [11,12] that phage display is the method of choice for projects requiring the rapid generation of antibodies to a large number of proteins. Making recombinant binders further offers added value from the instant availability of the gene encoding the antibody, for example to be used for gene function knockdowns. The generation of a renewable resource of 30,000+ monoclonal antibodies to the entire human proteome is no longer just a vision but can be achieved within a few years.

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