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Review

Perspectives for systematic in vitro antibody generation

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Abstract

After the completion and refinement of the human genome, the characterization of individual gene products in respect of their functions, their modifications, their cellular localization and regulation in both space and time has generated an increased demand for antibodies for their analysis. Taking into account that the human genome contains ~25,000 genes, and that their products are found in different splice variants and produce proteins with post-translational modifications, it can be estimated that at least 100,000 different protein products have to be investigated to gain a complete picture of what's going on in the proteome of a cell.

Antibodies are preferred tools helping with the characterization and detection of proteins as well as with elucidating their individual functions. The generation of antibodies to all available human protein products by immunization and/or the hybridoma technology is not only logistically and financially enduring, but may prove to be a difficult task, as quite a number of interesting targets may evade the immune response of experimental animals, for example, allosteric variants dependent on fragile interactions to cofactors, highly conserved antigens etc. For this reason, alternative methods for the generation of antibodies have to supplement these approaches. In vitro methods for antibody generation are seen to offer this capability. In addition, they may provide a cost effective and large scale production alternative for detection reagents for the research community in their own right. Among in vitro techniques, phage display has been evolved as the most efficient option for tackling this problem and approaches optimised for automation are emerging.

Maximum benefit for proteomic research could be generated by judicious and preferably international coordination of the ongoing efforts to combine the strengths of the well established animal based approaches and the novel opportunities offered by in vitro methods.

Keywords: Proteomics; Phage display; Ligand; Panning

1. The challenge of the functional genomics era

The last decade of the 20th century was dominated by the introduction of high-throughput technologies for genomic research. The application of robotics for liquid handling, clone picking and arraying, polymerase chain reaction (PCR) and DNA sequencing had a tremendous impact on the pace of data acquisition. As a direct consequence thereof, sequencing of many prokaryotic and eukaryotic genomes, including the human genome, has been accomplished (Lander et al., 2001; Venter et al., 2001) and recently refined (International Human Genome Sequencing Consortium, 2004), yet many more efforts are on the way.

The genome organisation and the gene content of these organisms mirror the incredible diversity of nature, from prokaryotes to eukaryotes. The genotype defines the species of an organism, the proteome defines the phenotype. By different interpretations of the same genetic content, dramatic phenotype variations can be achieved, like those between caterpillar and butterfly. Therefore, to determine the role of each gene, tools are needed to investigate the amount, localization and function of its protein products. Despite rapid increase of knowledge, around half of the open reading frames (ORF) identified by bioinformatics tools encode potential protein products with sequences not related to

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anything known and completely unknown biological functions — not to mention the very large fraction of sequences with a homology suggesting their function, waiting for experimental verification. The elucidation of gene function on a large, genome-wide scale will require systematic analyses of gene products but in turn provides a link between the sequence and the phenotype (Blackstock and Weir, 1999).

2. Antibodies for functional genomics

The sequencing of the human genome and the accessibility of many of the corresponding gene products and proteins through large clone collections have given a significant impetus to the application of molecular-recognition reagents in functional genomics. While this class of reagents is very diverse, including nucleic acid aptamers, affinity peptides and artificial binders based on scaffold molecules (reviewed in: Rimmele, 2003; Ulrich et al., 2004; Nygren and Skerra, 2004), here, we focus only on antibodies, as they are used in almost all currently ongoing programs. The use of antibodies will be a valuable asset for relating function and pathology to genomic sequence in biology and medicine. These approaches gained significance through increasing availability of systematic analytical methods requiring antibodies, for example, protein expression profiling by high-throughput immunohistochemistry (Warford et al., 2004). As a result, there is an intense interest in applying antibodies to functional genomics and proteomics, paving the path to a better understanding of disease processes. Further, antibodies will facilitate the discovery of new biomarkers and the development of diagnostic tools for the early detection of diseases.

Once the challenges of high sensitivity and high throughput are fully met, antibody arrays may allow the detection of abnormal protein expression profiles in pathological states and eventually accelerate disease-related investigations and drug discovery (Cahill, 2001; Hanash, 2003).

2.1. Antibody generation initiatives

As a consequence, several initiatives for the systematic generation of antibodies are already on the way worldwide. In a national endeavour, the Swedish Human Proteome resource (http://www.hpr.se/) is pursuing a program to generate a protein expression catalogue in different human tissues and organs. For this purpose, affinity purified polyclonal sera specific to human gene expression products are generated. The basic principle of this approach carried out by Mathias Uhlén and co-workers is to use Protein Epitope Signature Tags (PrEST) as antigens. Here, by utilising bioinformatic tools, protein sequences of 100–150 amino acids with relatively low homology to other proteins are identified and cloned as fusion proteins for production in *Escherichia coli*. The proteins are purified under denaturing conditions and used in mass immunisations. The resulting polyclonal sera are then affinity purified on individual PrESTs, yielding monospecific agents in respect of the antigen, and thereby avoiding polyreactivity problems frequently observed with polyclonal sera. Finally, the obtained antibodies are used for immunohistochemistry in tissue arrays, and the data are integrated to obtain a more global view on protein expression patterns as, for example, for all chromosome 21 encoded genes (Agaton et al., 2003, 2004).

In Japan, at the Kazusa DNA Research Institute in Chiba, Osamu Ohara and co-workers have already generated approximately 800 polyclonal antibodies against a large set of proteins in the ROUGE database (Rodent Unidentified Gene-Encoded Large Proteins; http://www.kazusa.or.jp/rouge/index.html), derived from ~1500 mouse counterparts of human KIAA proteins (mKIAA). The ultimate goal of the project is to accelerate the elucidation of the functions of KIAA/mKIAA corresponding gene products in the brain. All the antibody-derived immunohistological data is collected in a new Integrative Gene and Protein expression database (InGaP; http://www.kazusa.or.jp/ingap2) where it is linked with data from gene expression profiles using cDNA microarrays (Koga et al., 2004a,b).

Furthermore, the Human Proteome Organisation (HUPO; http://www.hupo.org) has united several animal based antibody initiatives in which it wants to generate a large number of mainly monoclonal antibodies against different human sub-proteomes such as blood plasma, brain and liver proteins. Within these different projects the generation of up to ~1000 antibodies has been reported (Hanash, 2004). For example, in the Human Liver Proteome Project (HLPP) a set of monoclonal antibodies against mitochondrial proteins will be generated, and in the Human Plasma Protein Project (HPPP) a monoclonal antibody bank with currently ~110 entries has been presented at the HUPO 3rd Annual World Congress (2004). These projects differ from the ones mentioned before by following the approach of identification of these antigens based on their expression locus and profile. Even a mouse monoclonal antibody bank covering the entire proteome (EPI) is conceived. Animal based methods have yielded most of the research antibodies currently in existence, and are the basis of the majority of funded programs.

2.2. Limitations and challenges

The ongoing antibody initiatives mentioned above represent a substantial contribution end encouraging start towards the systematic analysis of parts of the human proteome. However, a number of problems still have to be solved. Most of the antibodies reported by the above mentioned projects so far result from individual rabbit sera. Without doubt, quite a number of experiments can be performed with the amount yielded by one rabbit, but these sera are not an unlimited resource.

Another question to be tackled within the very near future is similar to a problem that arose in early stages of the human genome project. Currently, most of the antibody programs run on national level and it would be desirable to coordinate these efforts in the future to obtain antibodies against as many different gene expression products as possible. This channelling of efforts will have to be finely tuned within the collaborating antibody generating initiatives and will need to focus on three points: (1) to reduce work redundancy by ensuring a good information, data and material flow between participating facilities, (2) to stand for the delivery of antibodies of standardised quality, and (3) to introduce and define community standards for efficient exchange and compatibility of antibody application derived data. Ongoing initiatives on genome annotation and proteomics data demonstrate both the power and the difficulties of such a task. The lesson to be learned for the antibody generation initiatives is simple: to start as early as possible with a respective coordination.

A third problem cannot be handled by bioinformatics or coordination meetings, as it originates in the immune system itself. All of the aforementioned methods for the generation of antibodies are expected to fail to provide antibodies to quite a number of functional proteins. This can be a result of a number of reasons. First, if the immune system does not recognise an antigen as immunogenic, no antibodies will be made (Hayhurst and Georgiou, 2001). This happens for example to very conserved sequences, and is a particular problem when monoclonals to mouse proteins are attempted to be made. With mice being the most widespread mammalian model both in basic research and in clinical development, this problem is severe and has to be tackled.

Another type of antigens which usually fails to yield antibodies in animals are proteins which require a particular conformation. Usual immunisation procedures, denaturate proteins, and immunogenicity require the processing into short peptides, so an allosteric variant resulting from a fragile cofactor binding easily escapes immunogenicity.

An answer to fill the gap of knowledge for all of these non-immunogenic antigens is offered by methods which require neither immunisation nor the processing by an immune system. These methods, in the following summarised as in vitro methods, solely rely on the mere binding reaction to select a specific antibody. No antigen processing, no MHC presentation, no helper cells are needed to yield specific antibodies, thus releasing the boundaries set by immunogenicity in animals (Winter and Milstein, 1991). Antibodies to toxic compounds, lethal pathogens or highly conserved antigens can be obtained. Further, by controlling the biochemical milieu during this in vitro antibody selection, denaturation can be avoided or even allosteric variants can be induced by adding additional factors during the very step of selection. A nice example that can be achieved with these methods is the generation of an antibody to a conformational variant of a small GTPase (Nizak et al., 2003).

As an extra bonus, in vitro methods allow selection protocols which can easily be broken down to short experimental steps with any time of storage between each step, and which can be handled in the highly parallelised formats developed within the human genome project. This qualifies the in vitro methods particularly for upscaling by the use of automated parallel handling of the selection steps (Holt et al., 2000a,b; Walter et al., 2001). Further, most antibodies made in vitro are of human origin, offering the additional benefit of compatibility to mouse and/or rabbit antibodies in co-staining/co-localisation experiments.

The caveats and benefits of polyclonal sera and monoclonal antibodies, as well as the methods to obtain them, have been thoroughly discussed and documented during the last 30 years. In this review, we therefore look onto the knowledge that has been accumulated on in vitro generation of antibodies, with particular respect given to the challenges of producing an antibody depository for proteome research.

3. In vitro antibody generation

All the recombinant antibody selection techniques are based on affinity enrichment and on the simple but extremely effective concept of linking the phenotype with its genotype, i.e., linking the protein function with its corresponding gene. This is best demonstrated by surface display technologies, such as cell surface display on yeast cells (Feldhaus and Siegel, 2004), bacteria (Daugherty et al., 1999) and viral particles — mainly bacteriophage display (Hust and Dübel, 2005). However, alternative methods are in use as well where the DNA and the protein are hold together in vitro either non-covalently — such as in ribosome display (He et al., 2004) — or covalently, such as in mRNA display, e.g., puromycin-display (reviewed in Lipovsek and Plückthun, 2004) or the only recently developed covalent antibody display, where the protein and the corresponding DNA fragment are covalently fused by the bacteriophage P2 endonuclease P2A (Reiersen et al., 2005).

Nevertheless, among these methods, the most commonly used selection technology for recombinant antibodies selection technology until now remains display the M13-based on filamentous bacteriophage (Fig. 1). In the future, however, the alternative approaches of generating recombinant antibody molecules may well supplement or replace these strategies once brought to a comparable maturation (Bradbury, 2003; Bradbury et al., 2004).

Based on the wealth of experience with antibody phage display, at least two antibody initiatives have already started to employ in vitro methods for the systematic generation of antibodies for proteome research. Within the National Genome Research Network of the German Government (http://www.ngfn.de), the Antibody Factory (http://www.antibody-factory.de) has very recently started to develop technologies planned to allow the setup of streamlined processes optimised to yield recombinant antibodies for proteome research. The

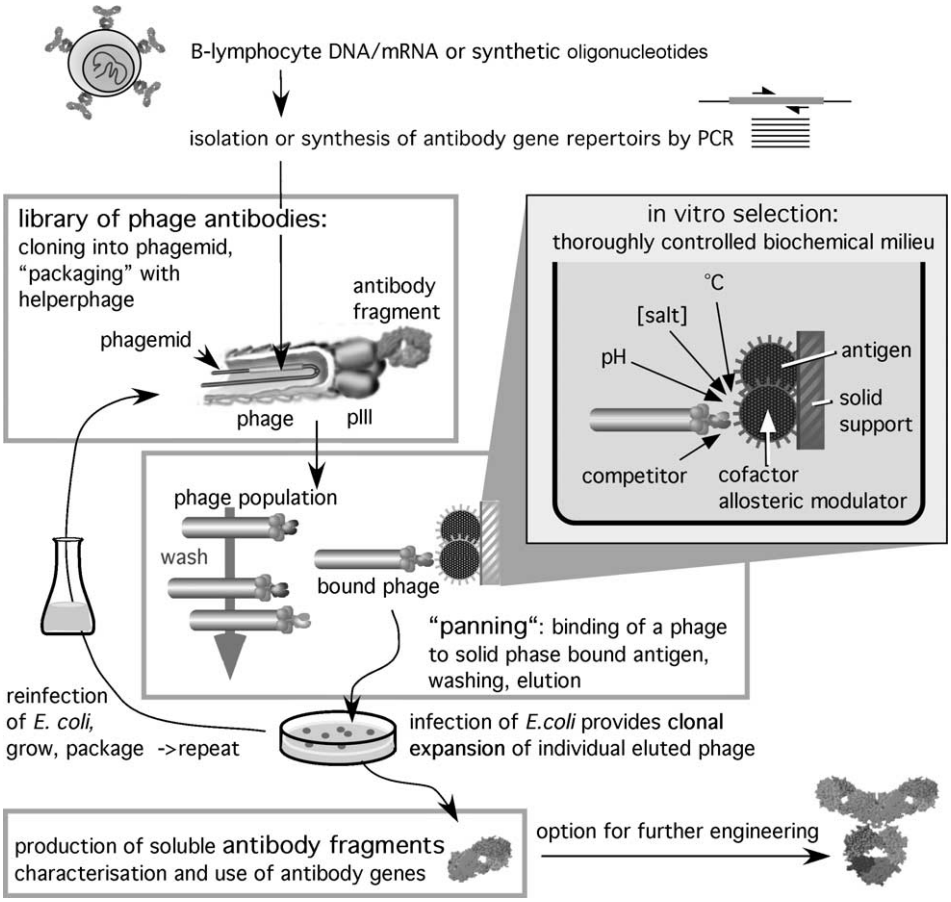

Fig. 1. Phage display allows to isolate antibody fragments by their binding function from large (10^6–10^{11}) antibody gene fragment repertoires. The biochemical conditions at the very moment of selection can be exactly controlled. Every antibody clone obtained with this method originates from a single molecular binding event of an antibody fragment to its antigen. However, its clonal expansion in *E. coli* provides an indefinite production source for the antibody and allows its engineering into other formats (whole IgG, enzyme or GFP fusions, diabodies etc.).

mission of the Antibody Factory is multifold. The project involves the generation of phage display antibody libraries, new selection procedures on polypropylene supports and approaches comparing different antigens, like bacterial expressed proteins or protein segments, as well as oligopeptides. Particular focus has been set from the onset to optimise the resulting products for the needs of the researcher, especially in respect of the handling by the "end user".

Already more advanced is the 'The Atlas of Gene Expression' project in the UK. John McCafferty and colleagues at the Wellcome Trust Sanger Institute in Cambridge (http://www.sanger.ac.uk/Teams/Team86/) have set up a pipeline for the systematic expression profiling of human proteins by immunohistochemistry and tissue arrays (Warford et al., 2004). For this purpose, they produce human protein domains in *E. coli* and use antibodies obtained by selections from highly diverse antibody fragment phage display libraries (Dyson et al., 2004).

3.1. Phage display

Both initiatives mentioned above utilises a very similar method for the selection of the antibodies. They rely on highly diverse antibody gene repertoires, which can functionally be screened by presenting them on the surface of a bacterial virus (phage display). Antibody phage display is based on the groundbreaking work of Smith (1985). Here, the genotype and phenotype of peptides were linked by fusing their short gene fragments to the minor coat protein III gene of the filamentous bacteriophage M13, resulting in the expression of this fusion protein on the surface of phage which allowed affinity purification of the gene by peptide binding. In a similar way, antibody fragments, fused to pIII, can be presented on the surface of phage M13 (McCafferty et al., 1990; Barbas et al., 1991; Breitling et al., 1991; Clackson et al., 1991; Hoogenboom et al., 1991). Regardless the production and presentation of complete IgG molecules (including the Fc part) are not possible due to limitations of the folding machinery of *E. coli*, this is not necessary as the Fv-part of an IgG provides the complete antigen specificity and can be easily produced in *E. coli*. For antibody phage display, two antibody formats are mainly used: the Fab fragment or the single chain Fv fragment (scFv). In an scFv, two polypeptides (VH and VL) are covalently connected via a peptide linker. The dominating system for the expression of the antibody::pIII fusion

proteins employs a plasmid with an autonomous replication signal, promoter, resistance marker and phage morphogenetic signal, allowing this "phagemid" to be packaged into assembled phage particles. Helperphage, like M13K07 (Vieira and Messing, 1987) or Hyperphage (Rondot et al., 2001), are necessary for the production of antibody phage as they supply the phage proteins not encoded on the phagemid. Due to a mutated origin, the helperphage genome is less efficiently packaged into phage than the phagemid, thus increasing the chances for the selection of the phagemid of interest during panning. A wealth of different systems for the display of scFvs or Fabs on filamentous phage have already been created (for review see Hust and Dübel, 2005).

3.2. Biopanning

The technique of in vitro isolation of specific antibodies by their binding activity is accurately called "panning", referring to the gold washers tool used to isolate a tiny nugget out of a huge pile of rubbish (Parmley and Smith, 1988). Here, the antigen is immobilized on a solid surface, such as nitrocellulose (Hawlisch et al., 2001), magnetic beads (Moghaddam et al., 2003), a column matrix (Breitling et al., 1991) or, currently most widely used, on plastic surfaces such as polystyrole tubes (Hust et al., 2002) or 96 well polystyrole plates (Barbas et al., 1991). The antibody phage mixture is incubated with the immobilized antigen, followed by thorough washing to remove the vast excess of non-binding antibody phage. The phage bound by their antibody moiety can subsequently be eluted (for example by using a protease site between pIII and antibody fragment) and the released phage re-amplified by infection of *E. coli*. This selection cycle can be repeated by infection of the resulting *E. coli* colonies with a helperphage to produce new antibody phage, which then can be used for a new round of panning (Fig. 1). The number of antigen specific antibodies is increased with every panning round. The enrichment of specific antibody clones in each panning round can be controlled by "polyclonal ELISAs" (Enzyme Linked Immunosorbant Assay). Usually 2–6 panning rounds are necessary before individual clones can be isolated.

4. Systematic generation of antibodies by phage display

Recombinant antibody technology using combinatorial libraries and in vitro screening technologies is well suitable for high-throughput antibody screening on a genome- or proteome-wide scale (Hallborn and Carlsson, 2002; Hust and Dübel, 2004; Walter et al., 2001). At the moment, however, proteome-wide applications are still not the major focus of most recombinant antibody engineering programs and the libraries and formats available are primarily optimised for the very different demands of human therapy (Hust and Dübel, 2004). Aiming at very different performance goals, these parameters need adaptation to the different demands of a streamlined selection process for research antibodies. Every individual part of the process has to be reconsidered and optimised. This concerns the antigen generation methods which have to be designed to match the throughput (and cost) of the antibody selection process, the selection of the appropriate antibody format optimised for the desired application, large-scale selection schemes and last but not least standardised characterisation and specificity validation.

4.1. Antigen availability

The availability of high quality target molecules is a necessary prerequisite for the setup of any successful antibody generation pipeline. The use of antigens individually purified from their naïve source is — despite their preferable superior quality — hardly applicable for parallelised antibody generation. The vast differences of protein structures and folding requirements, as well as their different states of modification and activation, render the setup of a generalised scheme for parallelised recombinant generation of antigen molecules difficult. Nevertheless, it's what we have at the moment. Several human clone collections of cDNA and full-ORF (open reading frame) expression clones have been established at non-profit resource centres within the Human Genome Sequencing Project, e.g., at the MRC gene service in Cambridge, UK (http://www.hgmp.mrc.ac.uk/geneservice/index.shtml), the Biological Resource Centre of the National Institute of Technology and Evaluation in Japan (http://www.nbrc.nite.go.jp/e/hflcdna-e.html), or the German Resource Centre for Genome Research in Berlin (http://www.rzpd.de).

4.1.1. Recombinant protein expression from cDNA libraries

Large-scale structural genomics initiatives, for example, the "Protein Structure Factory" (http://www.proteinstrukturfabrik.de), have established various supplementary expression host strategies for the expression of recombinant protein molecules. A major advantage of using recombinant selection antigens is the possibility for in-frame fusion to tag sequences, which facilitate their folding, purification and their attachment to panning support materials. Oriented binding is understood as a measure to reduce partial denaturation and improve epitope accessibility.

Despite undeniable restrictions in it's capability to make and/or fold larger eukaryotic proteins, *E. coli* still is the most prominent expression host in functional genomics and proteomics for it's ease of use and low cost. However, only ~20% of human proteins can be expressed as soluble proteins using currently available routine protocols. This percentage can be somewhat increased by the use of different tags that promote folding and solubility (Shih et al., 2002; Scheich et al., 2003). A recent study using 125 mammalian proteins revealed several protein features that correlated with soluble protein expression, and others that

do not. Not too surprisingly, a correlation between the yield and the molecular weight, the number of contiguous hydrophobic residues and low complexity regions, was found. There was no relationship between successful expression and protein pI, grand average of hydropathicity (GRAVY), or sub-cellular location. Further, the positive effect of solubility tags was confirmed, with Thioredoxin (Trx) and maltose binding protein (MBP) leading the list (Dyson et al., 2004). By allowing denaturation, protein expression in *E. coli* is more efficient and, for example, expression of ~50,000 individual clones of human proteins in parallel was achieved based on clone filters (Büssow et al., 1998). This arrayed cDNA expression library has already been used as a resource of proteins for the in vitro generation of recombinant antibodies (Walter et al., 2001).

Although denatured proteins can be and have been used as target molecules in vitro antibody selection experiments, soluble and, hence, more homogenous target structures are preferred. By using correctly folded antigens, it is feasible to select antibodies recognizing different epitopes and protein conformations, which will be of particular value for functional genomic studies. This possibility is a particular advantage of in vitro generation of recombinant antibodies when compared to conventional animal immunization procedures.

Eukaryotic hosts well established for parallelised expression of recombinant proteins are yeasts. Production protocols in microtitre plates have been developed for *Saccharomyces cereviciae* (Holz and Lang, 2004) and in *Pichia pastoris* (Böttner et al., 2002). Despite the glycosylation patterns in yeast are considerably different due to the presence of high mannose chains, many human proteins can be obtained as soluble proteins and in a post-translationally modified form in yeast.

Parallelised production of human proteins also is possible in insect cells (Albala et al., 2000). For this purpose, the protein coding genes can be cloned into the baculovirus system, which permits infection of various insect cell lines. Shuffling antibody ORF's from selection to production systems is facilitated by dual expression vectors allowing expression in *E. coli* as well (Chambers et al., 2004).

A significant and powerful option is the use of cell-free expression systems in which the proteins are expressed in an in vitro coupled transcription–translation reaction. These are usually based on either *E. coli* cell lysates (Kim et al., 1996), wheat germ cell lysates (Madin et al., 2000) or reticulocyte cell lysates (Craig et al., 1992), although other systems based on *S. cerevisiae* (Tuite et al., 1980) and *Drosophila melanogaster* (Scott et al., 1997) have also been reported. Some of these systems have been optimized for high-throughput genome-wide applications (Sawasaki et al., 2002; Busso et al., 2003). For example, the Riken Structural Genomics Initiative in Japan (http://www.rsgi.riken.go.jp) produces target proteins almost exclusively in bacterial cell-free expression systems (Yokoyama, 2003). Hence, this option is also particularly interesting in combination with recombinant antibody generation by phage display.

4.1.2. Protein fragments

The aforementioned methods aimed to produce full-length antigen from every gene, if possible in its naive conformation, as the use of soluble antigens dramatically increases the positive outcome of phage antibody selections. The available data, however, shows that this goal is still out of reach. By accepting this restriction, many strategies concentrate on using protein fragments in exchange for a better throughput. For example, Agaton et al. (2003) have used bioinformatic tools for the determination of unique protein epitope signature tags of human proteins, PrEST's, and after cloning and expression of the recombinant molecules with a histidine-tag and an albumin binding domain, they were purified and used for the immunization of rabbits. However, although non-expressible protein regions, like trans-membrane domains, can be omitted, the success rate for protein expression and purification under denaturing conditions was 76%. These strategies do not offer to select for native conformations but still require protein production and purification efforts similar to those for antigens generated from full length cDNAs. This can be avoided in a completely synthetic approach by using chemically synthesised peptides based purely on bioinformatic analyses of human gene sequences. In this case, sets of peptides can be predicted to be accessible at the surface of the protein (Wodak and Janin, 1978). Additional algorithms may be employed to avoid potential cross-reactivity to other gene products, to identify epitopes shared by mouse and man or within gene families etc., finally resulting in a robotic panning process requiring nothing more than the input of sequence accession numbers. Without doubt, not every antibody raised against PrEST's or peptides will bind to the naïve form of the protein; however, countless examples of polyclonal antibodies generated against peptides in rabbits and mice have been described and prove the validity of this approach. Further, unsatisfactory results of the peptide approach observed in the past may well be alleviated by the possibility of using large numbers of different peptides for each antigen in a parallelled panning approach. In addition, the success rate of epitope prediction may be improved by the generation of systematic data obtained for the first time in the currently started parallelised studies. Furthermore, by using, for example, phosphorylated variants, peptides can facilitate the search for post-translational modifications. Finally, by employing peptides synthesised in array formats on solid phase (Frank, 2002; Frank and Dübel, in press) for phage panning (Bialek et al., 2003), a significant reduction in numbers of experimental steps may yield an improvement of throughput that by far exceeds current capabilities.

No matter which antigen source is finally favoured, any proteome-wide antibody project faces the challenge that the logistics needed to generate antigens easily equals or

exceeds the efforts required for the antibody selection process itself.

4.2. Phage antibody libraries and formats

Dependent on the source of the antibody repertoire used for the library generation, the libraries are considered to be naïve or synthetic, or combinations of both. Naïve libraries are constructed from light and heavy chain repertoires isolated from non-immunised donors. For example, naïve libraries consisting of the repertoire of human IgM genes isolated from peripheral blood lymphocytes (PBL) (Marks et al., 1991; Dörsam et al., 1997) and from bone marrow or tonsils (Vaughan et al., 1996) have been constructed. (Semi-) synthetic libraries can be derived from unrearranged antibody genes of germline cells by cloning the CDR-containing gene segments of the different heavy and light chain families and rearrangement in vitro by PCR (e.g. Hoogenboom and Winter, 1992; Nissim et al., 1994). Other (semi-)synthetic libraries have 'targeted' diversity and consist solely of one or a few VH and VL frameworks and contain partially randomised CDR's. The diversity is introduced by PCRs with DNA–oligonucleotides having degenerated codons at desired positions. This procedure has the advantage that variations are only allowed in positions essential for antigen binding and by choosing well-expressed and well-folding frameworks (Pini et al., 1998; Knappik et al., 2000; Söderlind et al., 2000). Both naïve and semi-synthetic antibody libraries have yielded antibodies with KD's down to the sub-nanomolar range, so both types of libraries – or combinations thereof – can be considered as sources for the selection of antibodies for functional genomics.

Most of the human naïve libraries have been assembled to yield antibody fragments in the scFv format, however, there are also large naïve antibody phage display libraries made in the Fab format (De Haardt et al., 1999). ScFv-fragments are constructed by connecting the variable domains of the heavy and the light chains (VH and VL) by an amino acid linker of appropriate length (12–25 residues). ScFv's are small and usually well presented on the phage surface. A potential disadvantage is their lack of a structural support for VH–VL associations. As the VH–VL dissociation constants are within the low micro- and medium nanomolar range, their integrity is relying on the 'kinetic' stabilisation by the linker (Glockshuber et al., 1990), which does not prevent VH–VL dissociation but massively promotes re-association, thus rendering many scFv's susceptible to aggregation at higher concentrations. Fab fragments are comprised of the variable domain (VH) and the CH1 domain of the heavy chain and the complete light chain (CL). The stability of Fab fragments is comparable to IgG due a to natural disulphide bond covalently linking the two chains, and the additional contribution of CL–CH1 association. However, the presentation of Fab fragments on the surface of phage particles is usually not as efficient as for scFvs due to the necessity to produce and combine two polypeptides. However, from the viewpoint of a proteomic researcher, when compared to scFv's, Fab fragments offer the advantage not only to be more stable, but also to be more compatible to currently used assays without major adaptations to the protocol. They contain the entire light chain and a part of the constant region of an IgG heavy chain, so the standard secondary sera used for IgG detection can recognise them, while the detection of scFv requires specialised protocols usually employing additional anti-tag antibodies, a restriction applying as well to the multitude of alternative scaffolds for binding molecules, like anticalins, fibronectins, leucine-rich repeat proteins or ProteinA, among others.

4.3. Large scale selection schemes

There are various strategies for target presentation: proteins can be attached to microtitre plate surfaces (Krebs et al., 2001), to magnetic beads (e.g. by specific expression-tag sequences, Walter et al., 2001) or to immunopins in the 96-well format (Lou et al., 2001). They can be prepared by blotting 2D gels onto membranes (Liu and Marks, 2000; Liu et al., 2002), or represented by short oligopeptides (Hultschig and Frank, 2004). However, not all approaches are amenable for parallel selection and automation. For instance, selection efficacy on immunopins is limited by the rather small surface available for antigen presentation and lack of automation capability. Blotted 2D gels are spatially difficult to reproduce and the protein concentrations and modifications can strongly vary.

In the following, we focus on approaches proven in respect of automation. Countless immunological assays are based on microtitre plates and, hence, automation instruments for this format are commercially available. The setups can comprise individual liquid handling robots, ELISA washers, incubators and spectrophotometers. They are also amenable to phage display selections (Krebs et al., 2001).

Alternatively, antigens can be coated onto magnetic particles by using an automated pin-based magnetic particle processor (Kingfisher). Here, selections or ELISA can be handled in the 96-well plate format as well. The processor allows several positions for accommodating microtitre plates pre-filled with solutions for washing or incubation steps. Steps are performed by movement of the beads from plate to plate (Rhyner et al., 2003). An advantage of this approach is reduced background binding, as due to moving the beads, there are no remaining liquids in the system (Konthur and Walter, 2002). Further, problems arising from 'leaky' liquid handling systems can be avoided, not to mention the considerable cost savings by significantly reduced numbers of pipetting tips required in the process.

A new approach uses peptide arrays for phage display selections (Frank, 2002; Bialek et al., 2003). A significant advantage of this system would be that large numbers of different antigen peptides are directly synthesised in parallel

on the polypropylene folies, which are an appropriate support matrix for phage selections. Arrays of up to 2000 independent peptides have already been made, but their integration into an in vitro antibody generation pipeline still has to be implemented. New methods which may further facilitate antigen logistics are constantly emerging, for example, the in vitro synthesis of proteins from DNA spotted on arrays (PISA, He and Taussig, 2001), but have not yet been employed for antibody selections.

4.4. Characterisation of in vitro selected antibodies

The problem of "high-throughput" specificity validation of antibodies applies to both animal based and in vitro methods in a very similar way. Ideally, the antigen used for immunisation or panning should not be used for specificity conformation to prevent circle conclusions, which may, for example, hide the artefact of an antibody binding to a contaminant. However, in any application targeting systematic antibody generation in a proteomic scale, this cannot be achieved routinely, and a compromise has to be found. Current large-scale strategies for the screening of enriched phage display libraries are based on the concept of the arrayed library (Lennon and Lehrach, 1991). Using picking and spotting technology (Eickhoff et al., 2002), thousands of antibody-producing clones can be picked and gridded on membranes as bacterial clone arrays, and screened for specific binding with labelled selection targets (De Wildt et al., 2000). Most commonly, however, single colonies are picked into 96- or 384-well microtitre plates to generate antibody phage producing monoclonal cultures, and subsequent test of antibody specificity is done by enzyme linked immunosorbant assay (ELISA) on solid phase immobilised selection targets. This method is well adapted to the automated equipment pipelines of liquid handling robots, plate-washers and spectrophotometers. Modularised automated solutions have been developed for downstream evaluation of antibody selections. One station is comprised of a picking robot to generate single antibody expressing clones, another station is concerned with the antibody fragment production from bacterial cultures (100 microtitre plates in parallel; Hallborn and Carlsson, 2002). A large spectrum of variant methods has been developed for ELISA based specificity confirmation, including detection of soluble pIII::antibody protein (Mersmann et al., 1998) or even subcloning as fusion to alkaline phosphatase (Han et al., 2004), among many others. However, for a streamlined parallel application, addition of steps is always critical. A convenient way to confirm the binding of selected antibody fragments independent from the phage background is the use of the *sup*E system, which allows to switch from pIII-fusion to free antibody fragment (Griffiths et al., 1993). Parallelised ELISA has been demonstrated for 10.000 clones/day (Hallborn and Carlsson, 2002).

While microtitre plate-based ELISA screening allows 96 to 384 wells per plate, the use of protein microarrays allows an even higher throughput. The microarray-chip based method MIST (Multiple Spotting Technique, Angenendt et al., 2003) screens up to ~10,000 interactions at a time on a single chip, allowing parallel chessboard analysis of different antibody fragments for binding to different antigens, as well as to numerous negative control proteins. Here, the targets and the negative control proteins are spotted on a glass chip first. Then, after blocking, the antibody fragments are spotted on top of the first spots and detected with a secondary antibody (Angenendt et al., 2004). Similarly, selected recombinant antibodies can be screened for monospecificity on recombinant protein micro- and macroarrays (Lüking et al., 1999; Holt et al., 2000b). With increasing numbers of protein arrays available, and by using an intelligent selection of "non-binding" antigens comprising closely related proteins as well as possible contaminants, parallelised antibody validation may offer a significant decrease of "false positives". Individual specifically binding antibody fragments can also be subjected to kinetic analyses and affinity determination. This can be done for example by parallelised surface plasmon resonance in 96-well format equipment (for review see Malmqvist, 1999).

The selected antibody fragments can further be analysed on tissue microarrays, and automated setup for this were already reported (Warford et al., 2004). However, a true control of specificity has to employ two variants of every antigen. A solution which already could be achieved in automated form combines cDNA derived protein and synthetic peptides, with one used for panning, the other for specificity confirmation, or vice versa. It is however evident that careful control of the specificity of individual antibody preparations remains in the responsibility of the end user, as a true automated solution has yet to be established.

5. Conclusions and outlook

With the increasing awareness that many questions in the field of life sciences can only be answered by looking at multitudes of parameters at a time, there is also a growing demand for multinational resources and infrastructures, which can provide the research community with biological tools. In the case of antibodies, such an initiative offers significant benefits. For instance, it can pool all available data from the rapidly maturing national antibody initiatives, thus very much speeding up broad and systematic applications of the research agents by providing faster availability of both antibodies and research results. In contrast to other proteome related data, antibodies have the advantage of being established as robust research reagents for a long time, they have an easily to define small set of critical parameters and a good track record of standardization. As a result, dissemination and utilisation of knowledge and research agents can be expected to be much faster and more successful than with most of the other materials generated

or "standardised" in combined proteomics efforts. Further, by defining common future goals and monitoring and distributing the workload between international participants, redundancy is prevented and maximum benefit is generated from the national financial resources.

We believe that by combining the already existing well established methods plus optimizing the application of in vitro methods for the needs of proteomics, antibodies will have a major impact on the progress of functional genomics without being too demanding on another 'omics' matter — economics.

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