

Generation and validation of affinity reagents on a proteome-wide level

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There is a need for protein-specific affinity reagents to explore the gene products encoded by the genome. Recently, systematic efforts to generate validated affinity reagents on a whole human proteome level have been initiated. There are several issues for such efforts, including choice of antigen, type of affinity reagent, and the subsequent validation of the generated protein-specific binders. The advantages and disadvantages with the different approaches are discussed and the problems related to quality assessment of antibodies to be used in multi-platform applications are addressed. This review also describes the efforts to create a virtual resource of validated antibodies using a community-based portal and summarizes the status and visions for the publicly available human protein atlas (www.proteinatlas.org) showing the human protein profiles in a large number of normal and cancer tissues as well as a large set of human cell lines. Copyright © 2008 John Wiley & Sons, Ltd.

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INTRODUCTION

One of the great challenges in bioscience today is the need for well-validated probes to explore the human proteome. Such reagents can be used to analyze the corresponding proteins both *in vivo* and *in vitro* using assays, such as ELISA, protein arrays, Western blots, immunohistochemistry, immunofluorescence, and immune capture (Uhlen and Ponten, 2005) (Figure 1). The generation of antibodies on a whole proteome scale requires the selection and development of unit operations suitable for high-throughput production and this enforces the need to address strategic and technical issues, such as the choice of antigen, choice of antibody type, and the depth of validation of the generated affinity reagents.

The human proteome consists of approximately 23 000 non-redundant proteins (www.ensembl.org, Flicek *et al.*, 2007), defined as a representative isoform from every gene locus (Uhlen and Ponten, 2005) and it has been suggested that a vision for an international effort would be to generate paired antibodies to these proteins with a reasonable time frame (Uhlen, 2007). The paired antibodies should preferably be produced toward two separate and non-overlapping epitopes of the same protein target to facilitate the validation of specificity by allowing comparisons between the paired antibodies with regard to staining pattern across various analysis platforms. Paired antibodies also allow for coupled assays with high signal-to-noise ratios such as capture/detection (sandwich) assays and other assays with requirement for dual binding to increase specificity and sensitivity.

In this paper, we address the issues related to systematic generation of affinity reagents, including choice of antigen, type of affinity reagent, and the depth and breadth of the validation of the binder. The ultimate use of the affinity reagent is also addressed, since many applications involve denaturation of the protein targets, such as Western blots and immunohistochemistry, while other applications require epitopes in its native form. The advantages and disadvantages of various types of antigens and affinity reagents for systematic generation of protein-specific probes to the human proteome are discussed.

TYPE OF ANTIGEN

The choice of antigen is important and influences many of the subsequent operations, including the validation of the generated affinity reagent. The choices include "native" proteins, full-length recombinant proteins, recombinant protein fragments, and synthetic peptides. Another strategy is to use whole cells or whole tissue extracts, but such a strategy obviously yields a mixture of affinity reagents that need to be investigated later. Each type of antigen has its advantages and disadvantages depending on the final use of the affinity reagent. For example, antigens to be used to generate antibodies to be used for Western blot or immunohistochemistry do not need to be native, since the protein target in these assays will be partially unfolded, although the exact ratio of native and unfolded epitopes cannot easily be determined or predicted.

Native proteins can be obtained by protein purification from natural resources such as serum/plasma or tissue extracts. These antigens can be obtained with a correct native fold, which might be an advantage for efforts generating conformational epitopes (see below), although it should be noted that most immunization schemes, such as Freund's adjuvans (Freund, 1932), will denature the antigen prior immunization making arguments for a correct fold prior immunization less convincing. If native proteins are being used, it is also possible to obtain binders toward specific modifications on the protein target, such as glycosylation, and this might yield useful affinity reagents, although caution is needed since glycosylation is somewhat

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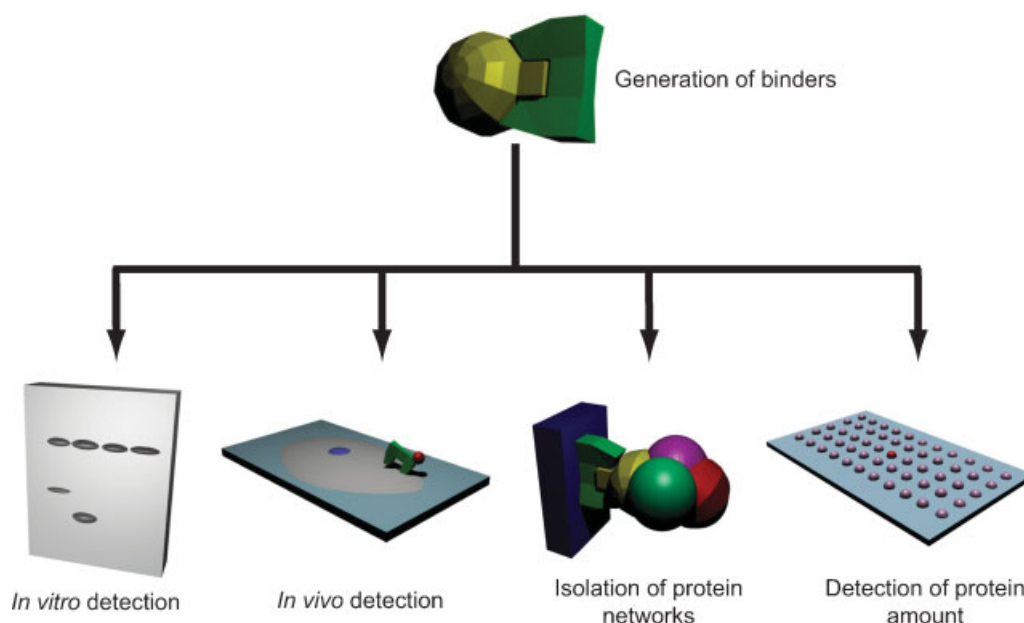


Figure 1. An attractive strategy for analyses and annotation of the proteome involves production of specific affinity reagents. Such reagents allow for documentation of expression patterns of a large number of proteins by western blot immunohistochemistry and protein arrays. Also more detailed analyses of the proteins by pull-out and mass spectrometry are possible, such as analysis of protein isoforms and specific interaction partners.

“generic” and yields similar carbohydrate structures on many host proteins.

Full-length recombinant proteins can be made in various heterologous hosts, such as *Escherichia coli*, yeast, *Pichia pastoris* or mammalian cells. The likelihood of correct fold will normally increase if an eukaryotic host is used, but many examples of correctly folded proteins generated in bacteria exist. There are several publicly available repositories of human full-length cDNAs (a summary of available libraries can be found on the web-page of The I.M.A.G.E. Consortium, <http://image.llnl.gov/>) and many expression systems for high-level production exist (Yin *et al.*, 2007). Large amounts of pure proteins can thus be produced facilitating both the generation of affinity reagents and subsequent validation. However, many proteins require protein partners or specialized cellular compartments, including membranes, to fold correctly and it is therefore not uncommon that cDNA expression from full-length clones yields partially unfolded proteins.

Recombinant protein fragments are a common strategy to generate antigens, since such fragments based on domains structure are often relatively soluble and easy to produce. The ease to generate clones by RT-PCR from RNA pools and sub-cloning into various expression vectors makes the cloning steps easy to carry out. It is also possible to avoid regions with high homology to other proteins taking advantage of the fact that many genomes have been sequenced and thus it is possible to use “sliding window” principles to search for regions with high or low homology to the entire relevant proteome. Recombinant protein fragments generated in this manner are called Protein Epitope Signature Tags (PrESTs) (Agaton *et al.*, 2003) and software to facilitate the selection of “unique” protein regions has been developed recently (Lindskog *et al.*, 2005; Andrade *et al.*, 2006). The PrESTs strategy allows for systematic expression of many thousands of antigens and the strategy has been used to express and purify more than 10 000 human protein fragments (Stenvall

et al., 2005; Steen *et al.*, 2006) by the human protein atlas program (Uhlen *et al.*, 2005; Persson *et al.*, 2006; Saviranta and Kallioniemi, 2006).

Synthetic peptides usually between 10 and 15 amino acids have also been frequently used to generate affinity reagents (Geysen *et al.*, 1984; Meloen *et al.*, 2003). Synthetic peptides normally consist of a single epitope and therefore subsequent epitope mapping is not needed. This means that if a polyclonal antiserum is affinity purified using the synthetic peptide as a ligand, an antibody fraction recognizing essentially a single epitope is, most likely, obtained simulating the specific single epitope recognition of a monoclonal antibody. The synthetic peptides route is therefore a frequently used strategy to generate antibodies in a streamlined manner and this has been further accentuated by the attempts to generate modification-specific affinity reagents, since the peptide can be synthesized with various chemical modification, such as phosphate groups. However, synthetic peptides can be expected to have lower success rates as compared to the recombinant protein fragments, since the epitope space delivered for immunization is much smaller. In addition, the flexibility of the synthetic peptides means that a large amount of entropy is lost at binding, making it relatively difficult to obtain high-affinity binders.

In conclusion, for large scale efforts to produce antigens for generation of affinity reagents, the two main approaches have up till now been the synthetic peptide route used by many commercial antibody providers and the recombinant protein fragments route, as exemplified by the human protein atlas effort (Uhlen *et al.*, 2005). Although conceptually attractive, the alternatives to generate full-length protein or isolate native protein with correct fold have the disadvantage that regions with high homology to other proteins from the same proteome will be found on the antigen. This limits such an approach to the generation of single epitope antibodies, such as monoclonal antibodies, and introduces the need to map the affinity reagent

Table 1. Type of affinity reagent

Type of affinity reagent	Origin (typical)	Number of epitopes	Size (kDa)
Polyclonal antibody (pAb)	Rabbit serum	Several	150
Mono-specific antibody (msAb)	Affinity-purified rabbit serum	Several	150
Monoclonal antibody (mAb)	Hybridoma cells	Single	150
Recombinant antibody fragment (scFab, scFV etc.)	Combinatorial libraries	Single	25–50
Scaffold binder (affibody, anchorin etc.)	Combinatorial libraries	Single	5–20
Nucleic acid-scaffold (aptamer)	Combinatorial libraries	Single	8
Peptides	Chemical synthesis	Single	1–2
Small chemical entities	Chemical synthesis	Single	<1

to ensure that it does not bind to regions of high homology to other proteins, thus giving rise to cross-reactivity.

TYPE OF AFFINITY REAGENT

There are many different types of affinity reagents available today and the major different variants are summarized in Table 1. Many thousand publications can be found describing the use of such reagents for biomedical research and again all have their advantages and disadvantages depending on their final use. The polyclonal antibodies are attractive mainly due to the synergistic effect obtained from the generation of a mixture of antibodies to several epitopes of the target protein. This allows more antibodies to bind to each target and also makes the binding assay less dependent on a single epitope. This is particularly important for antibodies to be used in multi-platform applications in which the protein target is denaturated in different ways such as detergent, alcohol, formalin, or mechanical stress. However, in polyclonal antiserum usually less than 5% of the antibodies possess specificity to the desired antigen and this means that cross-reactivity is often observed based on all the polyclonal antibodies to non-relevant targets. In addition, repeated immunizations to generate more antibodies often result in a new batch with certain differences in binding patterns. This makes polyclonal antibodies less attractive for applications in which a renewable resource of affinity reagents is needed.

The problems with cross-reactivity and batch-to-batch variation of polyclonal antibodies have led to the generation of mono-specific antibodies (Olive *et al.*, 2001), in which the polyclonal antibody mixture is affinity purified using the antigen as a ligand. This strategy to generate mono-specific antibodies has been most frequently used for synthetic peptides (Meloan *et al.*, 2003), but recently it has also been extended to antibodies generated by recombinant protein fragments (Nilsson *et al.*, 2005). The name “mono-specific” might be somewhat misleading since the affinity-purified antibody mixture often recognizes several epitopes, but the name reflects the fact that only antibodies specific for the antigen, and nothing else, are present in the antibody mixture. The use of mono-specific antibodies thus combines the synergistic effect obtained by having an affinity reagent recognizing several epitopes on the target with a defined procedure to remove antibodies not specific for the target protein.

Monoclonal antibodies, first described by Kohler and Milstein (1975) have become a corner stone in bioscience both as research reagents and for commercial applications, including diagnostics

and therapy. For therapeutic applications, the technology to generate “humanized” antibodies (Clark, 2000; Huston and George, 2001) has further improved these affinity reagents and such biopharmaceuticals are now a multi-billion dollar market. Advantages with monoclonal includes a defined epitope avoiding batch-to-batch variations and the fact that monoclonal antibodies can be made indefinitely and therefore is a renewable resource of affinity reagents. However, the single epitope makes monoclonal antibodies relatively vulnerable for applications in which the protein target is denaturated and many monoclonal antibodies are only functional in selected applications for which they have been developed. Thus, a validated antibody that works well in Western blots might not be functional in immunohistochemistry and *vice versa*. This dependent on a single epitope limits the use of monoclonal antibodies for systematic analysis in multiple applications in which the protein samples are denaturated in different ways. However, if a particular monoclonal has been shown to work in a wide variety of applications, this affinity reagent will be an attractive and renewable tool for future studies.

Recently, there has been a lot of development in the field of combinatorial libraries to generate a new generation of affinity reagents without the use of immunizations. Pioneered by Winter *et al.* (Clackson *et al.*, 1991), the use of phage display in combination with molecular biology techniques to create gene diversity has allowed a new generation of affinity reagents possible. This has led to the development of various recombinant antibody fragments such as scFvs, Fabs (Liu and Marks, 2000; Pini and Bracci, 2000; Söderlind *et al.*, 2001) and these can be combined not only to create bi-functional reagents, such as diabodies (Holliger *et al.*, 1993), but also to engineer antibodies with specialized effector functions or extended half-life (Presta, 2003; Kamei *et al.*, 2005; Schuster *et al.*, 2005; Petkova *et al.*, 2006; McEarchern *et al.*, 2007; Schlapschky *et al.*, 2007). However, the need for more robust affinity reagents or specialized features has also led to a new family of scaffold binders based on a protein scaffold in which diversity is introduced by the combinatorial approaches developed originally for antibody fragments. The first described protein scaffold was based on 58-amino acid three-helix-bundle domain of protein A (Uhlen *et al.*, 1984; Nilsson *et al.*, 1987; Gouda *et al.*, 1998) and called Affibody molecules, but recently many alternative scaffolds have been described (Nygren and Skerra, 2004; Skerra, 2007). Affinity reagents based on robust scaffolds can be very attractive for specialized applications, as exemplified by the development of an alkali-stable variant of protein A that can be treated with 0.5 M NaOH for 24 h without significant deterioration of IgG-binding activity (Linhult *et al.*,

Table 2. Application for affinity reagents

Application	Acronym	Epitope stage	Objective
Protein assays (ELISA, protein arrays etc.)	PA	Native (assay dependent)	Relative amounts
Western blots	WB	Partially unfolded (detergent)	Size analysis
Immunohisto-chemistry	IHC	Partially unfolded (formaldehyde)	Tissue profiles
Immunofluorescence (confocal microscopy)	IF	Partially unfolded (methanol, detergents)	Subcellular localization
Frozen tissue	FT	Native	Tissue profiles
Immuno-precipitation (capture, ChIP etc.)	IP	Partially unfolded or native	Isolation of complexes
Flow sorting	FACS	Native (cell surface markers)	Cell analysis

2004). Scaffolds based on nucleic acids have also been described and these aptamers have been shown to give high specific binding to many protein targets (Brody and Gold, 2000; Daniels *et al.*, 2003). The limited chemical space of the nucleic acids and the negative charge generated by the phosphate groups limits the use of the aptamers somewhat, but the fact that huge libraries can be constructed in a convenient manner has allowed the generation of high-affinity binders.

Finally, there are affinity reagents based on chemical synthesis, mainly driven by the drug development of pharmaceuticals. Many large combinatorial libraries of chemical entities have been constructed worldwide and automated systems for binding assays have been developed to allow the discovery of first generation binders that subsequently are developed further by drug development to improve pharmacokinetics and affinity (Roque *et al.*, 2004; Schuffenhauer *et al.*, 2005). These molecules usually bind in the active site of the protein target and therefore differ from most protein binders which often binds to the surface of the target.

In addition, combinatorial libraries of peptides have been constructed (Kay *et al.*, 2000) and binders to selected protein targets have been performed. However, the loss of entropy upon binding from these peptides makes them less attractive, as general binding reagent to protein targets. The attempts to generate specific peptide-based binders have therefore shifted toward protein scaffolds in which the diversity is created in a robust framework thus avoiding huge loss of entropy upon binding, thus allowing higher affinities in the nanomolar or even sub-nanomolar range.

In summary, most efforts to systematically generate antibodies have been based on immunological methods and the generation of polyclonal, monoclonal, or mono-specific antibodies. The development of combinatorial approaches to create affinity reagents has allowed a new generation of affinity reagents based on antibody fragments, protein scaffolds, or nucleic acid

scaffolds. However, so far, to our knowledge, no report for high-throughput production of affinity reagents has been published, although pilot studies have been described (Schofield *et al.*, 2007). The rapid development of new selection methods, such as ribosomal display (Zahnd *et al.*, 2007), bacterial display (Daugherty, 2007), yeast display (Boder and Wittrup, 2000), and protein fragment complementation assays (Michnick, 2003) makes it likely that high-throughput methods for generation of affinity reagent will be developed in the near future and these developments might cause a paradigm shift with the possibility to create an almost endless variety of affinity reagents to the human proteome in a streamlined manner. Until then, the most efficient way to generate affinity reagents is probably to use the streamlined methods for generating mono-specific antibodies using protein fragments or if a renewable resource is required to use the high-throughput systems for generation of monoclonal antibodies.

APPLICATION FOR AFFINITY REAGENTS

Affinity reagents can be used in many ways as probes to study the corresponding protein targets (Figure 1). The most common applications are summarized in Table 2. One of the most important issues for the use of a particular antibody is the folding state of the protein target in the different applications and a summary of these states is shown in the table. For protein assays, such as ELISA serum diagnostics, the protein target is often in a native state, although partial denaturation can occur during the sample preparation. Similarly, fluorescent assisted flow sorting (FACS) is often used on live cells and the proteins are displayed on the surface of the cells in a native form. In contrast, the proteins are denaturated using detergents sodium dodecyl sulfate (SDS) in Western blot applications and in immunohistochemistry, the proteins are fixed and denaturated using formalin. Similarly,

Table 3. Content of the human protein atlas

Version	Year	No. of targets	Total no. of abs	No. of mAb	No. of pAb	No. of msAb
1.0	2005	628	716	307	135	274
2.0	2006	1302	1514	500	254	760
3.0	2007	2471	3015	677	562	1776

The number of antibodies and protein targets in the human protein atlas (www.proteinatlas.org) stratified into monoclonal (mAb) and polyclonal (pAb) antibodies from commercial antibody providers and mono-specific (msAb) affinity-purified polyclonal antibodies from the academic human proteome resource (HPR) program.

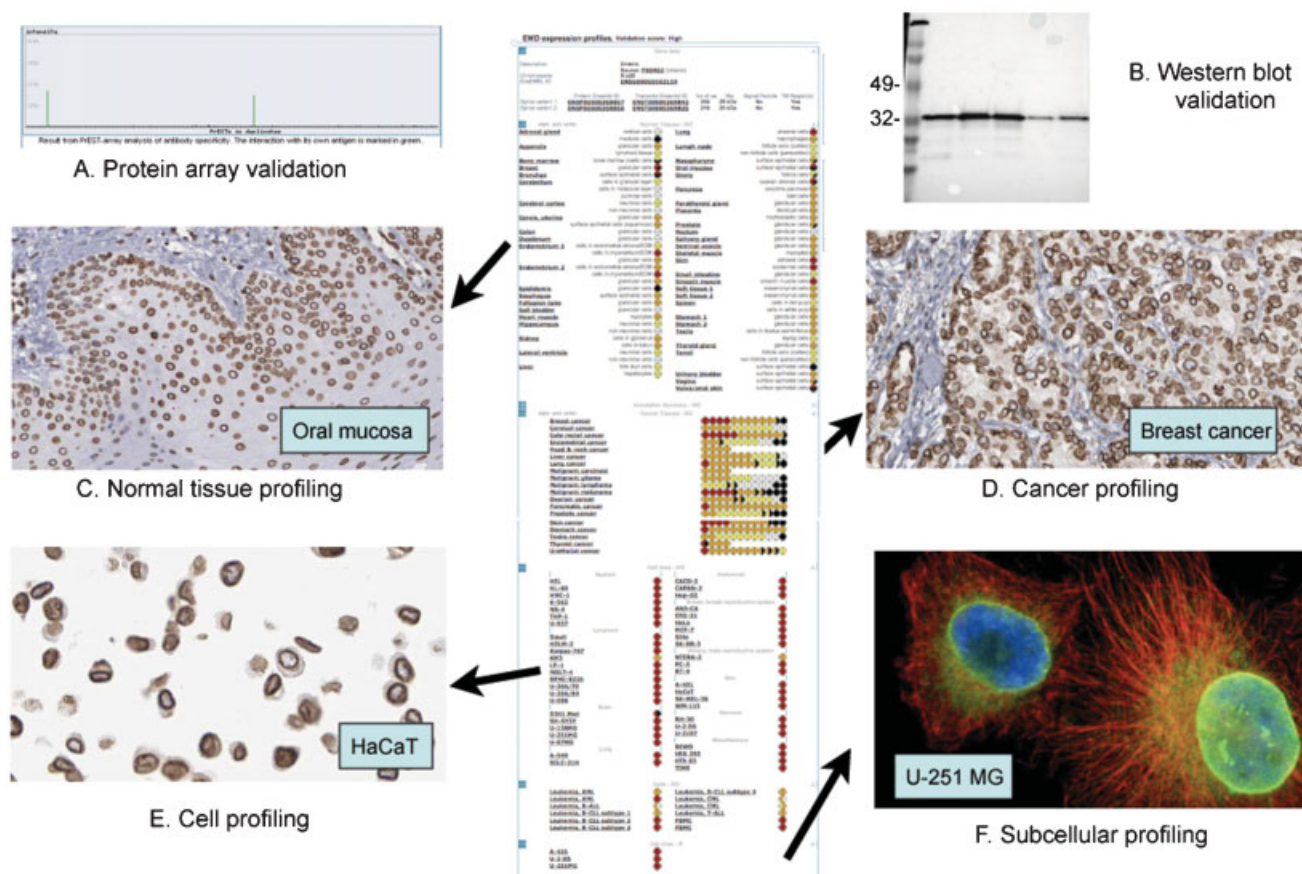


Figure 2. The human protein atlas. The portal (www.proteinatlas.org) shows the expression and localization of proteins in a large variety of normal human tissues, cancer cells, and cell lines with the aid of immunohistochemistry (IHC) and immunofluorescence (IF) images. The database contains validation data using protein arrays (A) and Western blots (B) for all the “in-house” generated antibodies and immunohistochemistry-based protein profiles for 48 normal tissues (C), 20 cancer families (D), and 48 cell lines and 12 cells (E). In addition, confocal images in three cell lines for some antibodies are shown (F). Each of the original images can be accessed through the tissue profile summary page and the gene, protein and validation data through the “antibody info” page.

proteins analyzed with immunofluorescence are often denatured using detergents (Triton) or methanol (Kononen *et al.*, 1998). For immuno-capture methods, the proteins are sometimes cross-linked prior to the immunoprecipitation operation, which might give rise to partially unfolded proteins, but other applications are carried out using native proteins. In summary, the protein target can have a large number of different folding states as a result of the particular application. This makes validation of affinity reagents difficult and emphasizes the need for application-specific validation.

CHARACTERIZING THE AFFINITY REAGENTS

Affinity reagents are usually characterized in two ways: (i) determining the binding parameters toward the protein target and (ii) mapping the binding region (epitope) of the protein target. Binding parameters can normally only be determined for affinity reagents binding to a single epitope, such as monoclonal antibodies, and the most common technique for binding measurements is surface plasmon resonance (SPR) in which

dissociation constants (K_d), including on- and off-rates (k_{on} and k_{off}) are determined (Malmqvist, 1999). Often the antigen is used as a ligand in these studies due to the difficulties to produce enough of purified protein target.

For affinity reagents generated by native or full-length proteins, it is also important to map the epitope(s) to establish which part of the protein target the affinity reagent binds. The antibody binding regions can be classified into linear (continuous) epitopes with a short consecutive stretch of amino acids and conformational (discontinuous) epitopes, consisting of segments that are distantly separated in the protein sequence and brought into proximity by the folding of the protein (Barlow *et al.*, 1986). The conformational epitopes are difficult to map and normally requires a three-dimensional structure determination of the binding complex, but some information can also be gained by alanine scans (Cunningham and Wells, 1989). The linear epitopes can be analyzed by several different techniques, such as overlapping synthetic peptides (Syu and Kahan, 1989) or random peptide display on phages (Lane and Stephen, 1993), but these methods are relatively cumbersome and many affinity reagents are therefore not mapped according to binding epitopes. Rapid and simple methods for epitope mapping are clearly needed and

it remains to be seen if new methods, such as the random peptide display on the yeast or bacteria can be used for epitope mapping on a whole proteome wide level.

VALIDATION OF THE AFFINITY REAGENTS

The validation of affinity reagents must normally be done in an application-specific manner. Unfortunately, almost all these validation assays are semi-quantitative and context-dependent and the results will be influenced by choice of tissue and sample preparation methods. The incredible dynamic range of proteins in human body fluids and tissues with dynamic ranges covering at least a dynamic range of 10^{10} (Anderson and Anderson, 2002) makes interpretations difficult. An antibody might show a correct and specific staining in a tissue with large amounts of the target protein, while showing cross-reactivity to another protein in a second sample, which contains only minute or no amounts of the correct protein. This problem enforces the need to define standards for affinity reagents validation and points to the need for standard operating procedures to carry out such validation.

The problems with validation of affinity reagents have led to the conclusion (Uhlen, 2007) that one of the objectives of an international effort to generate validated antibodies toward the human proteome should be to create a database to store information about the performance and quality assessment of publicly available antibodies from both academic and commercial sources. As part of the EU ProteomeBinder initiative (Taussig *et al.*, 2007), a database portal "Antibodypedia" has been developed (www.antibodypedia.com) with the long-term objective to store data about antibody validation. The rationale behind this community-based portal is to create a virtual resource of validated affinity reagents, with a focus originally on monoclonal and polyclonal antibodies. The idea is that antibody providers should be able to submit data on their antibodies with their own validation and that all data will be available for the public.

HUMAN PROTEIN ATLAS

A human protein atlas portal (www.proteinatlas.org) has been launched for protein profiles in normal and cancer tissues. The portal is publicly available without password protection. The first version of the atlas (version 1.0) was launched in August 2005 and contained 716 antibodies directed to 660 proteins (Table 3). More than 400 000 high-resolution immunohistochemistry images were published. A second version was launched a year later with an additional feature consisting of immunohistochemistry images of human cell lines and cells. The number of antibodies were doubled to 1514 and the number of images tripled to

1.2 million. The third version was launched in October 2007, again doubling the amount of antibodies to 3014 and doubling the images to 2.8 million. Three new features were introduced by this launch (Figure 2). First, the validation from the protein arrays and the Western blots for the "in-house" generated HPA antibodies were published, including the annotation of the validation results. Secondly, new confocal microscopy images of three cell lines using four fluorescent probes were released for more than 700 of the antibodies. This gives much more detailed data on subcellular localization and the plan is to release all antibodies with these data in due course. Thirdly, the new version of the atlas also contains an advance search function to enable complex queries about proteins expressed in normal and/or cancer cells. The vision is to extend this atlas with up to 3000 new validated antibodies per year to generate a draft version of the complete human proteome by 2015 (Uhlen, 2007).

CONCLUDING REMARKS

Several systematic efforts to generate protein-specific affinity reagents to explore the human proteome have recently been initiated (Uhlen *et al.*, 2005; Hober and Uhlen, 2007). The human protein atlas program has decided to focus its efforts on PreST fragments as antigen and affinity-purified (mono-specific) antibodies from rabbits as affinity reagent. This generates antibodies suitable for tissue profiling with high success rates, but the generated affinity reagents are not a true renewable resource, which limits their use for the broad scientific community. Other efforts have therefore been initiated, most notably the EU ProteomeBinder initiative (Taussig *et al.*, 2007), the NCI capture binding initiative (Haab *et al.*, 2006; Schofield *et al.*, 2007), and the Protein Atlas project at the Sanger center in England (Schofield *et al.*, 2007). The latter project is interesting due to its technical decision to base the generation on *in vitro* selection using phage display. In addition, thousands and thousands of researchers and companies are generating antibodies to different protein targets in a non-coordinated fashion. This emphasizes the need for an international coherent strategy to coordinate these efforts and to promote the generation of a preferable renewable and available resource of validated affinity reagents to the whole human proteome.

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