

## REVIEW

# Affinity reagent resources for human proteome detection: Initiatives and perspectives

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Essential to the ambition of characterising fully the human proteome are systematic and comprehensive collections of specific affinity reagents directed against all human proteins, including splice variants and modifications. Although a large number of affinity reagents are available commercially, their quality is often questionable and only a fraction of the proteome is covered. In order for more targets to be examined, there is a need for broad availability of panels of affinity reagents, including binders against proteins of unknown functions. The most familiar affinity reagents are antibodies and their fragments, but engineered forms of protein scaffolds and nucleic acid aptamers with similar diversity and binding properties are becoming viable alternatives. Recent initiatives in Europe and the USA have been established to improve both the availability and quality of reagents for affinity proteomics, with the ultimate aim of creating standardised collections of well-validated binding molecules for proteome analysis. As well as coordinating affinity reagent production through existing resources and technology providers, these projects aim to benchmark key molecular entities, tools, and applications, and establish the bioinformatics framework and databases needed. The benefits of such reagent resources will be seen in basic research, medicine and the biotechnology and pharmaceutical industries.

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## 1 Introduction

A major “post-genome” objective is to characterise fully the human proteome, including its composition, expression in tissues, cells and organelles, molecular nature and modifications, interactions, enzymatic functions, *etc.*, and to catalogue this information in health and disease. This enormous undertaking, currently ongoing in many centres, has extremely important implications for biological and medical science at many levels, from understanding individual cells

to systems, diseases and therapeutics. Two major approaches are the “classical”, nonhypothesis driven analysis of the components in complex protein mixtures by MS and, in contrast, “affinity proteomics”, in which individual proteins are identified by means of affinity reagents (also designated as binding molecules or simply “binders”) of known specificity. These methods are essentially complementary and indeed may be combined, *e.g.*, in immunoprecipitations (IPs), where the affinity reagent is a tool to copurify interaction partners of a specific target, which are then analysed by MS.

Affinity methods for protein analysis are employed universally. Their versatility provides information which may be qualitative, quantitative, static or dynamic, and which for proteomics includes determination of protein expression levels, localisation and function. In principle, for an affinity-based approach to make full use of the human genome information, comprehensive, characterised and standardised collections of specific binding molecules are required as reagents, directed systematically against as many of the

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**Abbreviations:** **CPTI**, Clinical Proteomics Technologies Initiative for Cancer; **IHC**, immunohistochemistry; **IP**, immunoprecipitation; **IUP**, intrinsically unstructured peptide; **NCI**, National Cancer Institute; **PrEST**, proteinEST; **RCA**, rolling circle amplification; **TMA**, tissue microarray;

individual proteins and their variant or modified forms as possible. Such collections should be made available as open access (though not necessarily cost-free) resources or infrastructures for the scientific community as a whole. The molecular species to be included could encompass natural and recombinant antibodies, engineered protein scaffolds, peptides, nucleic acid aptamers and small chemical entities. The importance of such resources and the variety of reagent types contained in them are certain to grow in the future as proteomics studies increase in scope and detail. To maximise use of the affinity reagents, resources should also be closely involved in the development of high-throughput, multiplexed assay systems and look beyond basic research towards healthcare applications in diagnostics and therapeutics.

While a small number of established European national infrastructures and projects address aspects of such a project (*e.g.*, the Swedish Human Proteome Resource, [www.proteinatlas.org](http://www.proteinatlas.org); the German Antibody Factory, [www.antibody-factory.de](http://www.antibody-factory.de)), some international initiatives have been established recently to coordinate production and application of affinity reagents on a proteome-wide scale in different centres (Table 1). In Europe, the EC 6th Framework

Programme supports the ProteomeBinders consortium ([www.proteomebinders.org](http://www.proteomebinders.org)), with 25 European and 2 US partners, while the National Cancer Institute coordinates a binding reagents initiative focused on cancer research (<http://proteomics.cancer.gov/>).

In this review we discuss aspects of creating resources for affinity-based, human proteome characterisation and what they should include.

## 2 Components of an affinity reagent resource

The goal of creating a comprehensive affinity reagent resource for analysis of the human proteome requires integrated consideration of several, complementary issues. (i) The targets for immunisation or screening, including epitope selection and the nature of the antigen, *e.g.*, full length protein, protein epitope signature tags (PrESTs), peptides, *etc.*

(ii) Access to large scale protein or epitope resources, *e.g.*, cDNA clones, recombinant expression systems, peptide production, *etc.*

**Table 1.** Current initiatives in affinity proteomics resources

Initiative	Aims, funding, scope
ProteomeBinders <a href="http://www.proteomebinders.org">www.proteomebinders.org</a>	Developing an implementation strategy for an open-access proteome-wide resource of affinity binders EC-funded FP6 Research Infrastructures Coordination Action, 1.8 M€, March 2006–2010 MAbs, pAbs, recombinant antibody fragments, alternative protein scaffolds, nucleic acid aptamers, small molecules Systematic pooling of expertise from 27 leading labs (25 Europe, 2 US)
Human Proteome Resource (HPR) and Human Protein Atlas (HPA) <a href="http://www.proteinatlas.org/">www.proteinatlas.org/</a> linked to HUPO antibody initiative <a href="http://www.hupo.org/research/hai/">www.hupo.org/research/hai/</a>	Generating a uniform collection of binding reagents against every human protein (nonredundant set), constructing a tissue atlas of protein expression Funding by Knut & Alice Wallenberg Foundation (25 M\$), Sweden Polyclonal, affinity purified (monospecific) antibodies; to date >1500 reagents; IHC on human tissue microarrays, normal and cancer; >1.2 mio images Encourages submission of antibodies from commercial/academic resources for inclusion in resource
Clinical Proteomic Reagents Resource within Clinical Proteomic Technologies Initiative for Cancer (CPTI) <a href="http://proteomics.cancer.gov">http://proteomics.cancer.gov</a>	Developing binding reagents against >1000 prominent cancer targets NCI-funded, budget 12.5 M\$ (total for CPTI 104 M\$), 5 year-programme from 2006 Main focus on mAbs Targets to be prioritised through literature record and community requests; reagent production to be outsourced to companies Data and resources (clones, hybridomas) to be publicly available
Antibody Factory <a href="http://www.antibody-factory.de">www.antibody-factory.de</a>	Developing novel recombinant antibody formats and panning procedures to facilitate selection and improve throughput Generating sets of antibodies against human transcription factors and signal molecules Four laboratories, part of the German “National Genome Research Network”

(iii) The nature of the affinity reagents themselves, *e.g.*, varieties of natural and recombinant antibodies, scaffolds, peptides, aptamers and small chemicals from combinatorial libraries.

(iv) High throughput binder production and selection methods, ranging from conventional use of immunised animals for polyclonal antibodies or mAb to *in vitro* molecular display systems and screening procedures for recombinant binding molecules, together with designs for automation, miniaturisation and cost reduction.

(v) Characterisation and quality control methods (specificity, crossreactivity, affinity, activity in standard assays) and agreed standards for reagent validation. These should be developed into a recognised set of “gold-standard” quality criteria.

(vi) Design of binder-based technologies, including high throughput systems for multiplexed protein expression analysis, *e.g.*, protein, cell and tissue arrays.

(vii) Applications of affinity reagents in functional proteome analysis, *e.g.*, interactomics, intracellular knockdown, *etc.*

(viii) Bioinformatics infrastructure for storage and distribution of the information on the properties of the affinity reagents, their targets and experimental data on their use.

(ix) Resolution of intellectual property issues and involvement of the biotech industry at different levels.

(x) Conditions for access by, and distribution of the reagents to, the research community.

Once technical and intellectual property issues are solved, the creation of a resource holding a complete proteome-wide set of affinity reagents will be clearly a matter of a decade or longer, rather than a few years. In the meantime, the research community could gain much from a recognised initiative putting into practice improved quality control for commercial binding reagents, *e.g.*, through setting up independent quality control centres and annotating the results in a publicly accessible database.

### 3 What are affinity reagents expected to do?

In general, the properties required of affinity reagents in a proteomics context are diversity of target recognition, high individual specificity (low crossreactivity), a range of binding affinities (*e.g.*,  $10^{-6}$  to  $10^{-12}$  M), utilisation in quantitative as well as qualitative assays, and adaptability to many different assay designs, with the potential for high sensitivity, accuracy and speed. Affinity methods include the well-established techniques of ELISA, Western blotting, IP, FACS, immunohistochemistry (IHC) and affinity chromatography, together with newer highly sensitive protein detection methods with binders conjugated to DNA or fluorescent labels and high-throughput array-based methods. Single molecule detection capability is becoming a practical possibility, which will be particularly valuable for enumeration of complexes and dynamic measurements.

As far as numbers of reagents are concerned, it seems certain that at least two, and probably several more, binders against each target will be required for different applications, for several reasons. For specific detection of proteins, it is often advantageous to incorporate two independent recognition events, as in sandwich assays [1], though the extent to which these can be multiplexed is limited by crossreactivity [2]. Proteins often have multiple functional and structural subregions of interest, such as active sites, motifs targeted for modification, and the modifications themselves, any of which can be important epitopes (see below). It is also desirable to have reagents of diverse affinities against the same target. While high affinity is a favourable factor in many applications, it can also be problematic in processes that require dissociation of the target after the initial binding. For purification processes, for example, it could be advantageous to utilise binders of increasing affinity in a serial arrangement; similarly, for quantitative analysis of proteins on capture arrays, the affinities of the binding reagents should be related to the concentration of the analyte to be bound [3]. Hence, depending on the ambitions, as well as the assay methods to be adopted, single binders are likely to be wholly insufficient even for a given researcher's needs, and certainly for the broader community.

### 4 Proteome scale, epitopes and targets

An effort to annotate the human proteome as a whole is being undertaken by the UniProtKB/Swiss-Prot Human Proteome Initiative (<http://www.expasy.ch/sprot/hpi/>). When all alternative splicings and PTMs are included, the number of human protein species is estimated to exceed 1 000 000 [4]; this complexity is increased further by individual single amino acid polymorphisms (SAPs), some of which may be highly significant in protein function and disease. The ability to differentiate between protein isoforms and modifications is one of the challenges of pharmaceutical research and development. In order to meet the objectives of comprehensive proteome characterisation, the scale on which affinity reagents might be needed is potentially immense, far exceeding that of the original genome project. Clearly, in order to keep a resource project within the bounds of what is desirable, necessary and practicable, the proteins against which affinity reagents are to be raised will have to be prioritised on the basis of existing knowledge, predictions and requirements. One approach is to aim at providing full reagent coverage of known pathways, networks or systems in “hypothesis driven” mode, in order to complete and refine current data. The alternative is to produce large sets of binders methodically against the proteome with no reference to known functionality, or even favouring those for which no functions have been attributed, in line with the nonhypothesis driven, global approach of genome sequencing itself. An example is found in the human protein atlas project in which binding reagents are made to identify the tissue localisation of proteins without preconceptions [5, 6].

Whatever criteria for target prioritisation are adopted, the details of the form of the antigen used and the epitopes to be targeted depend very much on the nature of the affinity reagent and the intended application. In a systematic approach, the details of which elements of protein structure to focus on will be governed by proteome-scale epitope selection, in turn directly linked with what the reagents are required to do and how many independent ones are needed. Multidomain, multifunctional proteins are the norm in the human proteome, where over 90% of all proteins have at least two structurally independent segments and a sizeable fraction have more than 10 independently operating units (e.g., P53, CBP/P300, fibrillin-1). The intrinsically unstructured polypeptides (IUP) present an important contrast with the folded domains [7]. There are several varieties of IUP, estimated to comprise as much as 30% of the proteome; one of their roles is as repositories of “linear motifs”, short peptides embodying autonomous function independently of tertiary structure and probably the most abundant category of protein functional module. They are used for regulatory interactions and many are post-translationally modified. An estimate is that there are in the range of 100 000–300 000 such motifs in the human proteome [4], representing important targets for affinity proteomics. Occasionally, the binding capability of an affinity reagent may be influenced by changes in the functional state of its target protein. Careful epitope targeting taking into account sequence information about known or predicted sites of PTMs and protein interaction modules may contribute to avoiding this problem.

Some questions of epitope selection which a researcher might face and the choices are summarised in Table 2. Bioinformatics programs exist which can provide information to guide decisions, though currently researchers have to call upon many different sites to assemble the information. However, only a few of the many potentially relevant protein analysis tools are aimed at binder selection, e.g., the heuristic antigenicity/hydrophilicity algorithms that predict candidate linear epitopes selection by identifying peptides with good scores. Others, such as structure databases, protein architecture analysis tools and protein functional site tools, are potentially informative for epitope identification and selection but their output is not specifically targeted for this purpose. Tools in the area of protein architecture include two for reporting IUP (GlobPlot [8] and DisEMBL [7]), the Eukaryotic Linear Motif (ELM) [9] resource cataloguing short functional sites and the Phospho.ELM [10] satellite resource cataloguing phosphorylation sites. Combining these resources with publicly available globular domain databases (e.g., INTERPRO) and other protein architecture and functional annotation resources (UNIPROT, HPRD) can provide an overview of protein modules for integration with epitope selection and modelling algorithms. By networking bioinformaticians with reagent users and producers, it should be possible to define the requirements of a binder selection toolkit and link them into a unified proteomics pipeline.

Target generation must be integrated into the process of building a resource, being responsible for about half the cost of binder generation. Complete domains or full length proteins expressed from cDNA collections will be one regular

**Table 2.** Protein epitope selection choices

Questions at the bench	Answered through epitope targeting for	Applications and technologies
Does the binding reagent need to recognise the native protein?	Full length expressed protein	Protein–protein interaction studies (coprecipitation), protein expression (capture arrays)
Will a binder targeted to a globular domain be sufficient?	Complex epitopes from folded globular domains	Binders for families of proteins sharing domains
Are binders to the unfolded (denatured) globular domains also needed?	Nonnative linear epitopes	Monitoring of unfolded protein; Western blotting, IHC
Would a linear epitope, from natively unstructured polypeptide regions, be desirable?	Linear, natively unstructured peptide epitope	Targeting of functional linear motifs
Are binders against posttranslationally modified variants (e.g. phosphoproteins) required?	Epitopes with and without PTMs	Tracking of signaling events influencing PTMs
Can the binder distinguish splice variants?	Epitope in modules alternatively spliced in or out	Detection of splice variants
Should the binder interfere (agonistically or antagonistically) with a function of the protein?	Functional site as epitope	Stimulatory/inhibitory binders for membrane receptors; competitive inhibition of protein–protein interactions; intrabodies

source of target, and required for assay and validations, while alternatives are the (subdomain) PrESTs [11, 12] favoured by the Human Proteome Resource and shorter peptides for linear motifs or recognition of denatured targets. The choices will be driven by the epitope pipeline considerations outlined above. Even complete omission of target generation can be considered (DNA immunisation), but although genetic methods for binder selection are attractive, protein is still required at some stage for screening and quality control purposes.

## 5 Affinity reagents

### 5.1 Natural and recombinant antibodies

Antibodies are among the most powerful reagents for proteome analysis, and are certainly the best established and most widely used, having the potential to identify, validate and quantify proteins. They are indispensable tools in research, diagnostics, drug discovery and, in a growing number of cases, therapy. Implementing standards for quality control (see below) will be a particularly important aspect of their future application in proteomics. The varieties of antibodies and their derivatives range from intact polyclonal and monoclonal antibodies and their Fab fragments, to recombinant single chain constructs (scFv,  $V_H/K$ ) [13] and camelid single  $V_H$  domains [14, 15, 16]. In principle, any or all of these may be suitable for inclusion in a resource depending on the envisaged applications [17], but the desirability of renewability makes polyclonal antibodies (even when made monospecific [18, 19]) a less attractive proposition. Recombinant products also have the advantages of readily available sequence information, which in the future may well substitute for stores of the proteins themselves, and the possibility of further engineering as fusion proteins.

### 5.2 Alternative binder formats

Recently, it has proved possible to develop alternative (*i.e.*, nonIg) binding reagents, using protein, peptide and nucleic acid libraries and appropriate selection technologies. The molecular entities include a variety of protein scaffolds recruited as frameworks for protein engineering, such as protein A (affibodies), lipocalins (anticalins) and ankyrin repeat proteins (DARPin) [20, 21], as well as ssDNA or RNA aptamers [22], from which novel variants capable of selective recognition of target molecules can be obtained. Because of marked differences in origin, biophysical characteristics and postselection improvement possibilities among the different novel binder classes, it is expected that these formats will generate valuable reagents for molecules of different natures; they will complement antibody resources, even becoming a preferred choice in applications such as functional annotation, detection, intracellular knockdown, structural analyses and protein isolation.

Nucleic acid aptamers are a particularly interesting potential source of high affinity binders against many proteins, with some distinct advantages in production and

amplification. “Classic” aptamers are single stranded nucleic acids (ssDNA or RNA), with similar properties of protein binding to those of antibodies [22]. Rapid association and slow dissociation rates give them an unexpectedly high affinity. Moreover, in the more recent photoaptamers [23], a photo-reactive nucleotide is introduced by replacing thymines (or uracils) with bromodeoxyuracil, to enable aptamer-target crosslinking when exposed to light. The precise alignment required for covalent binding increases the specificity of the interaction as well as allowing for stringent washing procedures on arrays [24]. A significant advantage of aptamers is that they can be “distributed” simply as sequence information, from which the users can carry out the synthesis themselves.

Nevertheless, antibodies are likely to remain the first choice of binder in many cases, due both to their properties and wide familiarity. This being the case how can alternative binders obtain wider acceptance? Ultimately the nature of the reagent may be inconsequential to the user so long as it works; the requirements are primarily functional rather than structural. Therefore, a key task is to establish criteria for adoption into a resource of any particular binder type based on suitability for given applications.

## 6 Production and selection systems

After adequate quality assessment, preexisting binders can be incorporated into centralised resources. However, the huge number of commercially available antibodies collectively targets only a relatively minor fraction of the proteome. Clearly, to get even near coverage of the proteome, new binders do have to be made in very large numbers. mAb production is capable of high throughput, *e.g.*, using mice immunised with antigen mixtures in combination with automated array-based screening gives capacities of 100s *per* year [25].

The alternative to immunisation is the use of recombinant systems, and there are several reasons to favour this approach (Table 3 [26, 27]). The generation of recombinant binders to the majority of proteins hinges on two central technologies: libraries of synthetic or recombinant binders and methods to select specific molecules from them. For both aspects a variety of systems have been developed, but with a lack of consensus over which to use. Since all selection technologies cannot be applied equally well to all libraries, the two must be considered in conjunction. A variety of libraries of different binding molecules have been described, including recombinant or synthetic antibody fragments [28, 29] in different formats [30, 31], alternative engineered frameworks [32–34] and single stranded nucleic acids [35, 36]. The most advanced screening and selection methods with reported library selections are phage display [37], ribosome display [30, 38, 39], RNA display [40, 41], yeast surface display [42, 43], bacterial surface display [44, 45], and two-hybrid systems such as the protein fragment complementation assay (PCA) [46] and intracellular antibody capture [47]. Variables which can guide a choice of technology include

**Table 3.** Features of recombinant affinity reagents and selection systems

Range of scaffolds	Ig and nonIg derived Engineered scaffolds may provide additional properties, <i>e.g.</i> , enzyme activity, fluorescence; binding to the target may modulate these properties, providing an intrinsic sensor of binding Compatibility with disulphides depends on selection format
Libraries of binders	Sizes up to 10 <sup>12</sup> species Construction technology and effort depends on selection format ( <i>e.g.</i> , PCR for ribosome display; bacterial transformation for phage display) Some selection formats ( <i>e.g.</i> , ribosome display) enable continuous evolution through error prone PCR
Diverse selection conditions/criteria	Positive or negative targeting of specific epitopes Selection for functional properties of the identified binders ( <i>e.g.</i> , receptor activation or blocking, intracellular activity)
Clones of recombinant binders	Archivable, distributable sequence data Engineerable for downstream uses, <i>e.g.</i> , with tags for immobilisation or detection
Scope of resulting binders	Target recognition not limited by antigen processing and immune response: Targets include nonimmunogenic epitopes, toxic proteins, generic PTMs Monomeric affinities 1 nM–1 $\mu$ M Evolution allows affinity maturation to pM range
Further	Potential for high throughput and automation Reduced cost Reduce use of animals, avoid animal welfare issues

robustness, scope for automation, capacity for binder evolution, library size and construction, and acceptability of disulphide containing domains. There is a particular advantage in methods which enable the first-selected binders to be improved by a built-in evolutionary cycle of diversification and selection, as in ribosome display, which can also be combined with selection technologies lacking this ability, *e.g.*, PCA [34]. Some methods will require significant optimisation for a proteome-scale throughput. While all methods are in principle capable of high throughput selections and robotic methods are under development in some centres [48], steps towards automation have been largely generic, encompassing liquid handling and the measurement of binding, and a “hands-free” selection technology is not yet in sight. Cost will be an important consideration in any large scale binder production programme, so that engineering processes to require much less target protein and use of miniaturised systems to allow economically feasible production will be essential elements.

## 7 Reagent characterisation and validation

The process of generating antibodies and other affinity reagents is often not the limiting step in obtaining large numbers of useful molecules. Instead, a major bottleneck lies in the detailed characterisation required to select the best ones for a given task. A vast number of binders, especially poly-

clonal and mAb, are currently available commercially, often >100 for the same target, with insufficient and inconsistent characterisation or documentation of the differences in properties. While meta-catalogues listing reagents from multiple commercial providers (such as Biocompare) ease sourcing of reagents and cost comparison, they do not provide a meaningful assessment of the quality and performance of reagents. In a proteome initiative, validation of reagents is a central issue where both the selection methods and available binder formats, as well as each individual reagent, have to be quality controlled. While different types of binding molecule may have superior characteristics for defined applications, there are quality criteria that can be measured for all formats, the key ones being affinity, kinetic parameters (association/dissociation rates), epitope specificity, selectivity and cross-reactivity. The relative importance of each parameter is linked to the proposed uses, which also leads to divergent methods for assessment of reagents from different laboratories. A vital role for which resource centres will be ideally placed will be to establish reference criteria for binder quality control and validation, together with standardised methods, for adoption in both the research and industrial areas [6]. The methods will have to be capable of an appropriately high throughput. Microarray-based systems of characterisation, using peptide and protein arrays, allow detailed analyses of binding properties such as specificity, affinity and epitope to a large number of antigens in one reaction and often reveal shortcomings in commercial reagents [49, 50].

## 8 Binder-based molecular tools and applications

In general, the application areas of affinity reagents can be divided into protein detection and separation methods, and functional studies. Large sets of affinity reagents are required for comprehensive coverage of specific pathways, protein interaction networks, protein modifications, proteins expressed in fluids or cells in particular disease states, *etc.* Ultimately, the choice of a class of binders (clonal or polyclonal, recombinant, scaffold), will depend crucially on the contemplated applications and the technologies in which they will be employed.

### 8.1 Affinity methods

#### 8.1.1 Sensitive and specific protein detection

Protein detection assays depend on binding by single or more binders to individual proteins, with read-out coupled to mechanisms such as fluorescence, surface plasmon resonance [51], linked enzymes or DNA strands, *etc.* In addition to the well established methods of ELISA, Western blot, IHC and IP, there are emerging highly specific and sensitive techniques combining binder recognition with DNA, such as immunoPCR [52], immunoRCA (rolling circle amplification) [53], and proximity ligation [54] and, increasingly, multiplexing devices such as protein arrays. It will be important to optimise performance and standardise methods both for scientific and diagnostic applications. Criteria to be applied include the sensitivity of the procedures, dynamic range, specificity, throughput, and convenience of establishing and performing the procedures with new binders.

Sandwich assays using pairs of binding molecules for increased specificity are widely employed [2]. Proximity ligation offers an innovative range of assays, with the potential to improve sensitivity and specificity further, based on the conversion of protein detection to the formation of amplifiable DNA strands. Proximity ligation technology improves sandwich assays in two ways, by both greatly reducing the effect of crossreactions on the one hand and incorporating huge signal amplification on the other [54]. Binding molecules, which to date include examples of polyclonal and mAb and ssDNA aptamers, are conjugated to oligonucleotides designed to be ligated only when the correct pairs [54] or trios [55] of binders are brought together on the same target molecule. Subsequent to the DNA ligation reaction, the product in the form of a new DNA strand or circle can be highly amplified either in solution or *in situ*, by PCR or RCA. Proximity ligation provides the basis for highly sensitive and specific assays for a range of important targets, from cytokines to infectious agents [56, 57]. It has also been extended to visualisation and quantitation of individual intracellular protein complexes, where the affinity reagents target the interacting partners [58].

#### 8.1.2 Protein arrays

The development of high-throughput proteomic tools, based on principles of highly parallel analysis, is crucial to realising the goal of comparing healthy and disease tissues. Just as high-density DNA microarrays have allowed global genome analysis to be performed in a matter of hours, the same ability is now needed for proteomics. Protein array systems are becoming increasingly used for rapid, global comparative studies. In the current platforms, binders or target proteins are immobilised on surfaces [59], such as glass slides or beads [2], and used to detect and quantify interacting molecules. The assays are often highly miniaturised, providing rapid, economical and sensitive readout. Some of the current issues in protein array production are summarised in Table 4.

In one format, capture arrays carrying immobilised antibodies [60], antibody fragments [61], aptamers [24, 62] or other affinity reagents [63], are used to quantitate proteins in complex mixtures, applications including monitoring protein expression in tissues and fluids. In another design, arrays of target proteins or peptides are used to define binder specificity, epitope recognition and crossreactivity and for functional studies. There are potential medical applications of both array types in diagnostics, *e.g.*, monitoring of biomarkers or antibodies in plasma. In the example shown in Fig. 1, a photoaptamer array [24], in which target proteins become bound and then crosslinked to photoactivated ssDNA aptamers, is used to detect 100 human serum proteins in parallel. The covalent binding allows harsh washing conditions to be used, reducing background and increasing S/N, with the protein being detected by a general fluorescent stain.

Protein arrays are currently offered by a number of manufacturers and resource centres (<http://www.functionalgenomics.org.uk/sections/resources/index.htm>). Given the heterogeneity of the target proteins and the different means of producing [64] and using the arrays, standardisation is again an important issue. This includes the specific software tools and formats for reliably exchanging data. Standards for unambiguously representing protein and capture array data, should include guidelines for what minimum information is needed to describe an experiment (conditions, tissue, technique, protocol, protein targets, *etc.*), a basic data model, and formats for data representation and exchange compatible with existing standards efforts.

#### 8.1.3 Lysate arrays

Lysate microarrays utilise tissue or cell extracts to monitor protein expression [65]. This is an emerging technology for the simultaneous profiling of 100–1000s of cell and tissue samples for key protein targets and pathway status indicators [66]. General standards for array production, measurement and analysis protocols have yet to be established. Features requiring annotation include lysate preparation, printing

**Table 4.** Choices and issues in protein microarray production

	Alternatives/parameters	Optimisation criteria/aims
Array surfaces and coatings [97]	Microwells, modified glass surfaces [98], beads, NC, hydrogels	Capacity, background, spot quality, feature size
Molecules for immobilisation	Natural, recombinantly expressed (bacterial [99], mammalian, cell free [100, 101, 102]), engineered (tags, GFP-fusion)	Stability, modifications, folding, function
Immobilisation methods and conditions	Adsorption, covalent bonding, affinity interaction (biotin/streptavidin, His tags [103], Strep-tag) Protein concentration, time	Function, binding capacity, orientation, stability
Ligand binding conditions [104]	Ligand concentration, buffers, time, temperature, mixing	Binding of ligands with different physicochemical properties
Detection methods	Fluorescence (confocal scanning, planar waveguide), amplification methods (tyramide, RCA), label-free detection systems (MS, SPR [51, 105]), direct labelling or 'sandwich' procedures	Sensitivity, accuracy, specificity, signal/noise, dynamic range
Reduction of scale	Robotics, liquid handling and spotting technologies for microarrays, nanoscale arrays, single molecule arrays and microfluidics methods	Throughput, accuracy, sensitivity, cost
Data analysis, storage, sharing	Centralised or distributed resources	Establishing common standards [106], software for protein array data

methods and instrumentation, substrates, detection methods, imaging and data analysis. As in Western blotting and tissue arrays, the binding reagents are required to recognise denatured proteins, but unlike tissue arrays (below), lysate arrays are 1-D, providing no spatial information on localisation of individual proteins. Their advantages lie in giving a rapid overview of large numbers of samples, both for screening of targets and identification of useful binders, with good quantitation and without special interpretative skills. A current focus is in profiling of cancer cell lysates, to provide protein level validation of genomic and transcriptional level findings [67–69].

#### 8.1.4 Tissue arrays

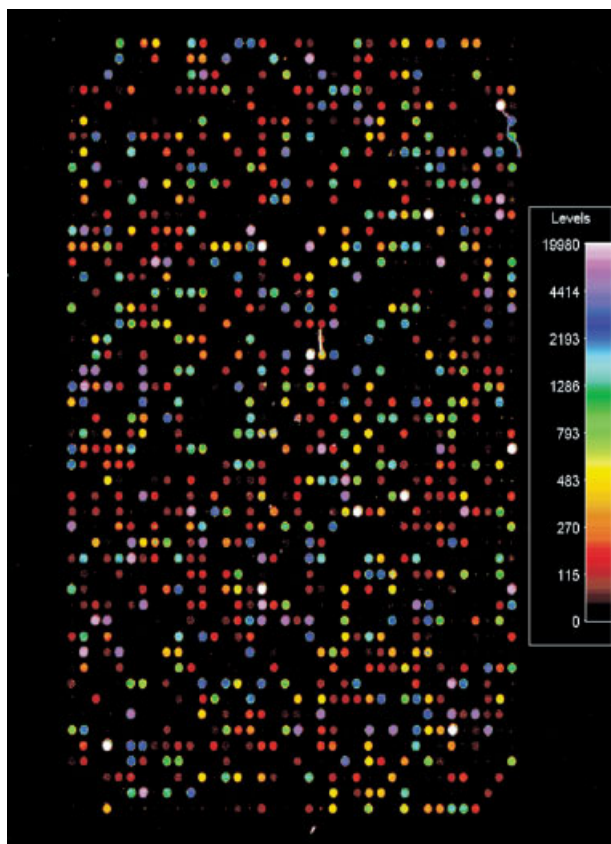
Tissue microarray (TMA) technology provides an automated high-throughput technique, in which up to 1000 paraffin-embedded tissue samples can be assembled into one paraffin block as an array [70]. This allows investigators to use a single slide to conduct controlled studies investigating binding on large cohorts of tissues, using only small amounts of reagents. The source of tissue is restricted only by availability in paraffin, while the small amount of sample that is needed minimises loss of unique or scarce tissues. Tissue biobanking resources will make TMAs widely available. For example,

the Human Proteome Resource has access to a biobank containing 3 million paraffin blocks of tissues and 50 000 fresh frozen tissues and cells, which is extensively used in the developing atlas of protein distribution, mapped systematically using hundreds of monospecific polyclonal antibodies ([www.hpr.se](http://www.hpr.se)). In an adaption of the TMA approach applicable to the study of cells (*e.g.*, *in vitro* cultured cell lines), microarrays of cells embedded in a gel matrix are a tool for antibody-based proteomics [71].

## 8.2 Proteomics applications

### 8.2.1 Proteome expression profiling

Particularly successful examples of proteome expression profiling are found in the proteome atlas projects, *i.e.*, the Human Proteome Resource [6, 72], where polyclonal antibodies are used to profile comprehensively protein localisation in human TMAs (above), and the mouse Atlas of Protein Expression, employing recombinant monoclonal fragments from phage libraries to carry out IHC on mouse tissues, focusing on cell surface markers (<http://www.sanger.ac.uk/Teams/Team86/>). In addition to standard IHC approaches, an automated high-throughput protein colocalisation approach ("multiepitope-ligand cartography"), can be



**Figure 1.** Serum proteomics assay on a photoaptamer array, detecting ~100 different human serum proteins (with replicates). Proteins bound to cognate aptamers were photo-crosslinked and stained with NHS-ALEXA 555, which labels lysines in the proteins. The image is false colour (see scale) with the dynamic range of detection corresponding to protein levels from pM to nM. (Image kindly provided by Larry Gold, SomaLogic.)

employed for mapping potentially hundreds of different proteins in one tissue section or cell sample [73]. It involves running sequential cycles of *in situ* staining with single fluorescence-tagged affinity reagents, imaging and complete bleaching, followed by re-exposure to further reagents. This enables identification of proteins which colocalise through complex formation or transient association.

### 8.2.2 Protein interactions and pathway analysis

Large-scale binder collections will also benefit functional proteomics in the contexts of protein–protein interactions (interactomics) and systems biology. Affinity isolation linked to MS of purified and enriched protein complexes allows the identification of unknown components. While generic binders, *e.g.*, antitag [74] or antiphosphotyrosine [75], have been successfully used for MS-analysis of protein–protein interactions and signalling networks, respectively, collections of specific binding reagents would allow the more extensive use of IP of endogenous, untagged proteins. Another widely used

application of binder sets is chromatin IP (ChIP) for isolation of protein–DNA complexes [76]. A particular requirement in chromatin and epigenetic studies is for antibodies against histones and their extensive and complex modifications. The identification of histone arginine and lysine acetylations and methylations is dependent on antibodies which are frequently poorly characterised and crossreactive, so that improved validation, *e.g.*, based on histone peptide arrays, is urgently needed. Well-characterised reagents are crucial for determination of epigenetic changes on a genome-wide scale, *e.g.*, in connection with human cancer risk [77].

Miniaturised, multiplexed immunoassays, in the form of capture [78] and lysate [65] arrays allow sensitive, rapid detection and quantitation of a high number of analytes from minute amounts of samples using sets of defined reagents. The use of antibody arrays to analyse complex pathways or systems provides a good example of the use of such reagent sets [60]. For the analysis of signalling pathways, both the expression level and functional state of signalling proteins are crucial components. For example, thematic antibody arrays comprising targets from functional contexts such as phosphorylation, cell cycle regulation, apoptosis and nuclear signalling were used to analyse changing protein expression levels in response to a differentiation stimulus [79]. Receptor phosphorylation and ensuing kinase cascades provide a prime target for multiparameter analysis of signalling and effects of signalling inhibitors, by either employing arrays of phospho- and pan-specific antibodies and incubating with cell lysate [80] or in the reverse format using an array of lysates of differentially activated cells [66]. Furthermore, activity-based enzyme profiling in which the functional state of enzymes on complex protein mixtures is detected by small-labelled probes has been parallelised on an antibody capture array [81].

Protein–protein interactions are crucial for the understanding of cellular signalling. A capture array of peptides corresponding to protein-domain binding motifs together with a panel of antibodies against signalling proteins has been employed to probe for signalling dependent protein–protein interactions [82]. In a batch method requiring simple addition of fluorophore-labelled antibodies to microlitre amounts of cell lysate, fluorescence cross correlation spectroscopy can be employed to detect signalling-dependent protein–protein interactions [83].

### 8.2.3 Functional proteome analysis and intracellular knockdown

While most applications of affinity reagents are concerned with extracellular targets, it is also possible to achieve intracellular expression in order to explore functional effects on regulatory and metabolic activities of living cells, in particular, binder-mediated protein modulation or knockdown. “Intrabodies” are single-chain recombinant binding molecules, often scFv or single domains, whose intracellular expression [84] can have effects similar to antisense or RNAi

and influence or destroy the functions of targeted proteins, with potential for functional proteomics. One problem to be overcome is the requirement for disulphide formation in Ig domains [85], which could be avoided by use of appropriate scaffolds. The ability to target proteins in specific compartments and interfere with specific motifs or PTMs gives intrabodies advantages over RNAi and similar whole-gene knockdown methods [86]. Applications in cancer, cell signalling, neurodegeneration and infection suggest that they are promising tools for therapy as well as basic research [87–90].

#### 8.2.4 Translation: from biomarker discovery to diagnostics and therapeutics

One driving force behind production of proteome-wide sets of affinity reagents is the search for protein signatures associated with particular clinical indications. To perform global proteomic analysis of biomarkers will require high-density arrays, containing thousands of probes in the form of strictly standardised binders, such as high affinity antibody recombinant fragments or photoaptamers. A focus is the analysis of malignancies [91]. Several different solid and haematopoietic malignancies, such as breast and colon [92] carcinomas, and certain nonHodgkin lymphomas, are still in urgent need of early diagnosis and improved therapy. Capture array technology, leading to biomarker and target discovery, will be a major area of importance of affinity reagents for the pharmaceutical and biotech industries, and one from which healthcare will benefit directly through increased accuracy of early detection, prognosis and staging of disease. One should also keep in mind future novel therapeutic modalities for immune intervention, using antibodies [93], intrabodies, scaffolds, or aptamers.

### 9 Bioinformatics framework for representation of affinity reagents

In order to facilitate sharing of information related to affinity reagents throughout the scientific community, the corresponding data must be standardised. For each type of reagent, it is necessary to identify the minimum information needed to unambiguously describe both the binder and its protein target, and to formalise this description as an ontology that will facilitate the exchange of information between different data systems. This description should include, as far as possible, knowledge about the properties of the interaction derived from experimental evidence. Since binder-target interactions can be seen as a special case of molecular interactions in general, the well-established PSI-MI XML (extensible markup language) format may be adapted for transferring information on binder properties [94]. A schema based on these specifications could be used to set up a central database of all known affinity reagents against the human proteome, containing essential infor-

mation about each reagent/protein pair, as well as links to distributed resources containing detailed descriptions of binders and proteins.

### 10 Reagent production initiatives in Europe and the USA

A well-formulated plan of implementation for the future expansion of affinity reagents is essential, building on the integration of existing infrastructure resources, with clear choices of technology, molecular platforms and mechanisms of access. In Europe some national programmes are underway, notably in Sweden (the Human Proteome Resource) and Germany (the Antibody Factory). However, the importance and scope of an affinity reagents resource exceeds the interest and possibilities of any individual country or company. The ProteomeBinders initiative ([www.proteomebinders.org](http://www.proteomebinders.org)) aims to integrate and coordinate these and other European initiatives, to create a durable European infrastructure that supports the systematic generation, application and exploitation of affinity reagents for academic, medical and commercial purposes [95]. The long-term objective is to provide access to affinity reagents and tools for future European concerted efforts within the life sciences and healthcare and to link to large-scale, research-driven projects in functional proteomics and systems biology. A parallel activity, the Clinical Proteomics Reagents Resource, has been set up by the National Cancer Institute (NCI) to develop principally mAb as reagents against targets specifically in the cancer area ([http://proteomics.cancer.gov/programs/reagents\\_resource/](http://proteomics.cancer.gov/programs/reagents_resource/)). This falls within the NCI's Clinical Proteomics Technologies Initiative for Cancer (CPTI) with the aim of providing reagents identifying proteins (especially those at low abundance) linked to cancer processes, in order to accelerate biomarker discovery, cancer diagnostics development, and therapeutics monitoring [96] (Table 1).

For practical purposes, it is imperative that the resources created by these initiatives be operated in a way which gains acceptance by both the academic and commercial sides of the scientific community. The ProteomeBinders consortium is well positioned in this respect, by including all forms of binders, represented in the project by researchers who are active in the area of binder production and selection. The NCI initiative will involve industry closely in reagent (mAb) production, through funding incentives, and will provide a web-interface to catalogue, describe and aid distribution of the reagents. The discussion of whether affinity resources should be publicly funded or developed on a more commercial basis is similar to that which pervaded the human genome sequencing project. What is important is that they be well coordinated and do not develop into a disorganised “free for all”. There is also the issue of sustainability: the products generated within or on behalf of a consortium should be available long-term, beyond the duration of most funding grants, which will inevitably require a cost for access if the resource is to be self-sustaining.

## 11 Conclusions

The development of large-scale affinity binder resources will have a major impact in many areas. They will help to develop the potential of genomics applications in life sciences research. Moreover, binding reagents are relevant to progress in the diagnosis and treatment of diseases that dominate healthcare in developed countries, particularly cancer, autoimmune and inflammatory diseases. Therapy with binding molecules is an area which will grow intensely over the coming years, building on the success of antibody therapeutics such as Rituxan (Rituximab), Humira (Adalimumab), Remicade (Infliximab), Herceptin (Trastuzumab), Xolair (Omalizumab) and Avastin (Bevacicuzumab) [93], as well as the aptamer Macugen, and others now entering the clinic. Other beneficiaries will be the high-tech postgenomic biomedical, pharmaceutical and biotech industries. In short, the scientific and economic importance of affinity binder-based initiatives are substantial, both for the near and the more distant future. Although the goals are ambitious, there are no fundamental technological reasons why they cannot be achieved, but future efforts must be thoroughly coordinated and organised. Given the numbers of binders required, an internationally coordinated action would seem to be the only way to tackle this tremendous but feasible task, the successor and similar in scale to the human genome sequencing project and indispensable to deriving the full benefit from the vast of genomic information now available.

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