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General Information

Deliverables

- imaGenes Protein Macroarrays: 22 cm x 22 cm PVDF membranes presenting up to 2 x 27,648 in situ expressed proteins
- imaGenes Protein Macroarray Manual
- Technical Data Sheet “Scoring Template for Macroarrays”
- Annotation Table

Shipping and Storage

imaGenes Protein Macroarrays are shipped at room temperature. The Arrays should be stored dry, light-protected and at room temperature. Under these conditions storage for at least 4 months is possible.

Product Use Limitations

imaGenes’ Protein Macroarrays are developed, designed, and sold for research purposes only. All due care and attention should be exercised in the handling of any of the materials described in this text.

Quality Control

imaGenes is certified according to the international norm ISO9001:2008. This ensures that all individual steps in imaGenes Protein Array generation and handling are carried out according to the most stringent quality standards. As part of the quality assurance program, the manufacturing process and performance of the resulting imaGenes protein arrays is monitored routinely to ensure highest performance and reliability. Detailed information about the quality control procedures is available upon request.

Technical Assistance

For any questions regarding imaGenes’ Protein Array products or services, please contact our Product Support Department.

Phone +49 (0)30 9489 2468
Email product-support@imagenes-bio.de
Application Areas of Protein Arrays

Introduction

Protein arrays constitute a powerful tool for high throughput and multiplexed protein analysis, including protein detection, investigation of protein interactions with various types of molecules and determination of protein functions. Protein array technology is highly sensitive, highly parallel (multiplexed), generates large amounts of data in a single experiment with comparatively low sample consumption, and therefore is highly economical. They facilitate the parallel screening of thousands of interactions, encompassing protein-antibody, enzyme-substrate, protein-ligand or protein-drug and protein-protein interactions.

imaGenes’ Protein Arrays

imaGenes Protein Macroarrays, made of tens of thousands of recombinant proteins derived from selected human tissues, allow screening experiments on:
- Target protein identification, using antibodies/serum/plasma
- Screening of human serum/plasma for auto-antibodies
- Epitope mapping of antibodies
- Functional assays, e.g., phosphorylation, methylation, ribosylation
- Identification of DNA/RNA binding proteins

imaGenes provides Protein Arrays as stand-alone product, for direct use by the researcher. Alternatively, we offer full services for epitope mapping or human serum/plasma auto-antibody screening on Protein Arrays.

imaGenes’ high-density Protein Arrays offer a convenient and robust option to perform such studies in a highly cost effective way. They have been used successfully in a large number of scientific endeavors (see ‘References’), some of which are described here in more detail.

Protein-Protein Interaction Analysis

Proteins mediate their biological function through interactions with other proteins. Therefore, the systematic identification and characterization of protein-protein interactions (PPIs) is considered a key strategy to understand protein function and to uncover the complex organization principles of functional cellular networks. The number of possible contacts between protein surfaces is astronomical. In this context, protein array technology opens up new avenues for the characterization of putative proteins and the identification of molecular partners involved in metabolic and regulatory networks in cells. Protein arrays have distinct advantages: they allow the direct detection of protein-protein interactions and can be used to monitor different parameters such as relative protein concentration, binding affinity and the influence of protein modifications or mutations. A further application is the identification of domains and amino acids involved in known protein-protein interactions.

In a recently published approach, high-density Protein Arrays were used to identify new potential regulators of integrin function in platelets and to put them into their biological context using information from protein-protein interaction (PPI) databases. In this process, biotinylated synthetic peptides corresponding to a highly conserved signature motif in the functionally critical cytoplasmic tail of the integrin α-subunit was used to probe the Protein Array for high affinity interactions (Raab et al., 2010).

The usefulness of Protein Arrays in simple and sensitive high throughput interaction screening studies of human proteins, which does not require radioactivity or expensive equipment, was demonstrated by Grelle et al. (2005). Here, GST-tagged human fusion proteins were expressed in E. coli, and after native lysis with lysozyme, crude protein extracts were prepared under non-denaturing conditions in 384-well plate format. The crude bacterial cell extracts were used for incubation overnight with the high density spotted filter
membranes. PPIs were detected subsequently by an anti-GST antibody mediated staining reaction. Many novel interaction pairs were identified and subsequently confirmed by in vitro Protein-Protein Binding Assays (e.g. pull-down, two-hybrid and co-immunoprecipitation experiments).

An additional example for the effective use of imaGenes’ Protein Arrays is a screening approach with proteins or protein fragments that contain or lack posttranslational modifications, to uncover differential protein interactions (Kowenz-Leutz et al., 2010). Also, they have been successfully employed in a study monitoring enzyme activity at a high throughput scale. This was done by performing solid-phase supported enzyme reactions, e.g. an in situ methylation of the filters by the recombinant methylase PRMT and the methyl donor [^3H]AdoMet (S-adenosyl-L-methionine) (Lee and Bedford, 2002).

Protein-Antibody Interaction Analysis

High-density protein technology is a fast and effective means for determining the specificity of antibodies and can be used to further improve the accuracy of antibody applications. As large numbers of potential antigens are analyzed in parallel, the methodology allows identification of a primary antigen for a particular antibody as well as the determination of potentially cross-reacting proteins. Moreover, it was demonstrated that detection of specific interactions is possible even if unpurified antibodies (e.g. hybridoma supernatants) were used directly in screening experiments (Kijanka and Murphy, 2009). Therefore, the method may also prove useful for the quick and cost-efficient assessment of antibody specificity and cross-reactivity during antibody manufacturing.

The Protein Array screening can be used to identify highly specific antibody-antigen interactions and eliminates the need for many rounds of selection using animal immunization or in vitro techniques, such as phage or ribosome display. Thus, the method can be applied to the high throughput isolation of specific antibodies against many different targets in the human proteome (Holt et al., 2000). Furthermore, large Protein Arrays provide a unique opportunity to identify representative, but highly complex antibody profiles that discriminate between symptomatic cohorts of appropriate patients (Kijanka et al., 2009). Screening Protein Arrays with body fluids, such as serum, plasma or cerebrospinal fluids (CSF) from large numbers of patients with an autoimmune disease does not only allow the identification of potentially novel auto-antigens, but also potentially assists the prognosis, diagnosis, and subtyping of autoimmune diseases. Additionally, profiling the auto-antibody repertoire can help to elucidate the pathophysiology of auto-immunity, enabling novel treatments. In general, all of the identified auto-antigens are potential diagnostic markers. Such characterized auto-antigens are suitable candidates for different diagnostic and therapeutic applications (Gibson et al., 2010).

Additionally, the protein arrays can be used for epitope mapping. The redundant nature of the cDNA expression library represented on imaGenes’ protein arrays allows identification of the epitopic region and determination of possibly shared epitopes in cross-reacting proteins (Murphy et al., 2010; Kijanka and Murphy, 2009). Furthermore the protein sequences of the corresponding clones can be used to design different peptides to find the specific epitope recognized by the antibody (Cepok et al., 2005; Grelle et al., 2005).

Protein-DNA/RNA Interaction Analysis

Many DNA/RNA binding proteins are involved in the regulation of gene expression and maintenance. While DNA arrays allow DNA binding sites in genomes to be identified, Protein Arrays can help to identify proteins that bind to DNA/RNA targets of interest. Several proof of principle studies have been described (Mahlknecht et al., 2001).

Advantages of imaGenes Protein Arrays

- As > 10,000 different proteins can be simultaneously analysed, Protein Arrays represent a powerful alternative approach for identifying potential binders in a large scale format. The arrays provide an extensive coverage of the human proteome.
- The main advancement of the array technique over existing technology is its high-throughput link between DNA sequence information and protein expression.
- Because all clones have already been sequenced, the identity of a protein recognized by a new antibody or another protein is
immediately evident without the need for further sequencing, allowing a rapid identification of binding interactions.

- Due to the sequence information most of the proteins represented on the array were annotated to a particular gene, facilitating a biological interpretation of the results (e.g. by performing a pathway analysis).

- If the arrays were screened to characterize the specificity of an antibody, sequence information can be used to gather information on putative epitopes shared between cognate antigens and non-cognate proteins showing cross-reactivity with the tested antibody. In this connection the amino acid alignment can be performed by means of web-based software (e.g. CLUSTALW, LALIGN) to define the potential epitope regions (Kijanka et al., 2009).

- Furthermore the sequence information for an identified protein enables the design of particular peptides which can be used in a peptide scan analysis to obtain high-affinity ligands (Cepok et al., 2005).

- One clear advantage of this approach is that expression clones identified as “positives” during a screening procedure can readily be retrieved from imaGenes. Thus large quantities of the recombinant protein can be produced for further experimental analyses, e.g. His-tagged recombinant human proteins can be purified using chelate-affinity chromatography, even in a high throughput format using 384 MT plates (Büssow et al., 2004). Related full ORF expression clones are also available from imaGenes, incl. the shuttling in the respective expression vectors.

- The proteins expressed and purified from the corresponding clones detected in a screening experiment can be used for the further validation and specification of the discovered interactions. For example after the identification of the molecular targets from a novel antibody the specificity and sensitivity of the interactions can be confirmed by Western immunoblotting and transfectant studies (Kijanka et al., 2009; Larkin et al., 2004).

- Based on the corresponding protein expression libraries, many different proteins can be easily expressed and purified in a large scale format to produce customized protein microarrays (Horn et al., 2006).
imaGenes Protein Macroarrays

Specifications

With E.coli expressed proteins derived from cDNA libraries from different human tissues (fetal brain, lymphocytes, lung, colon), imaGenes offers one of the largest collection of arrayed proteins for screening experiments. Clones for the production of Protein Arrays are sequenced and have undergone a stringent in-frame analysis. The expression vector adds a HIS-Tag to each expressed protein. All proteins on the arrays have been verified for expression by detecting this HIS-Tag using an anti-HIS antibody. EST sequences for the majority of the expressed proteins are provided along with the arrays. Protein expression clone collections and the respective individual cDNA clones are available for in-depth follow-up experiments such as structural genomics. imaGenes Protein Macroarrays consist of up to 27,648 spots, which are printed in duplicate (totaling to 55,296 protein spots) onto 22 cm x 22 cm PVDF membranes.

![Vector Structure of imaGenes protein expression clones](image)

Types of Macroarrays

imaGenes offers two types of Protein Arrays. The corresponding cDNA expression libraries were validated and advanced based on more than ten years experience in the field. To generate a protein expression cDNA library, total RNA was isolated from different human tissues and oligo(dT)-primed cDNA was synthesized by reverse transcription. The cDNA library was directionally cloned into a modified pQE (Qiagen) vector that allows IPTG-inducible expression of His6-tagged fusion proteins. Large numbers (usually more than 250,000) of individual clones of the library were subsequently arrayed in microtiter plates and gridded onto high-density arrays. A monoclonal antibody recognising the vector-encoded N-terminal RGSH₁₆ sequence of expressed proteins detected 20% of the library as putative expression clones which were re-arrayed into a smaller sub-library. For further validation of the sub-library the cDNA inserts were amplified by PCR and analyzed by 5’-tag sequencing. Based on the sequencing results we re-arrayed new clone collections. Thus, imaGenes offers the following Protein Array types as standard, readily available products:

- **hEXselect:**
  The hEXselect protein expression library is derived from human fetal brain and contains 24,000 clones. It comprises full-length as well as shorter cDNA clones in an E.coli expression vector. All clones have been 5’ tag sequenced and are fully annotated. The size of the low redundancy collection allows a complete spotting in duplicates onto a single Protein Array, which saves processing time and hybridisation probe volume. Note that the majority of the clones represent partial proteins, which may include artificial protein sequences corresponding to the 5’ UTR sequences, with one third matching to the human proteome (Büssow et al., 2000). Protein Arrays are delivered with an accompanying annotation table and respective spotting positions.
UniPEx:
The UniPEx protein expression library consists of 2 Arrays representing clones in 2 different vectors. In total, more than 100,000 sequenced clones from different protein expression libraries (human fetal brain, T-cells, lung) were analysed in depth for their coding potential. After in-frame analysis only clones with a confirmed in-frame ORF were selected and redundancy with respect to clones per gene was minimized (< 3 fold). In total, the 15,300 UniPEx clones are representing 7,390 distinct human proteins. The UniPEx set comes together with an annotation table showing cloneIDs per filter position, geneID, and gene description in Excel format with links to public databases.

Custom-made Protein Arrays can be generated from subsets of these libraries

imaGenes Protein Macroarray Production – a Short Overview

The fabrication of imaGenes Protein Arrays combines robust contact printing technology with in situ protein expression directly on the surface of PVDF membranes. Protein synthesis is performed in an E. coli based expression system for recombinant proteins. Expressed proteins are extracted and immobilized under denaturing conditions, directly on the PVDF membrane surface. Resulting imaGenes Protein Arrays mainly consist of almost linear sequence epitopes, ideally suited for epitope mapping, for auto-antibody assays, and for antibody cross-reactivity screenings. As many proteins are not completely denatured or partially renature under assay conditions, imaGenes Protein Arrays may also be applicable to the identification of DNA/RNA binding proteins, and to functional assays such as phosphorylation, methylation or ribosylation studies.

imaGenes' Protein Array fabrication process comprises the following steps:

1. Robot-assisted printing of re-arrayed cDNA clones that express recombinant proteins onto PVDF membranes and incubation overnight.
2. Induction of protein expression on PVDF membranes by incubation with IPTG.
3. Lysis and fixation of expressed recombinant proteins on PVDF membranes.
4. Quality Control

The following figure gives an overview of the imaGenes' Protein Array production process and the subsequent screening and analysis procedure:

![Diagram of Protein Array production and subsequent screening experiment](image-url)
Quality Control of imaGenes Protein Macroarrays

The manufacturing process of imaGenes' Protein Arrays is monitored stringently by in-process controls. Appropriate growth of bacterial cultures is checked and recorded for each production batch. After spotting, cultivation, induction of protein expression and fixation of proteins on the membrane, autofluorescence of the resulting arrays is scanned for a consistent and homogeneous spotting and growth pattern. At least one filter per production batch is tested by incubation with a mouse anti-His antibody and subsequent staining using an anti-mouse IgG/alkaline phosphatase coupled antibody with Attophos as substrate. Filters are scanned and assessed again for consistent signal patterns.

Protocol-Supported Applications for imaGenes’ Protein Arrays

- **Serum Screening:** Arrayed proteins are hybridised with (a minimum of 100 µl of customer-provided) human patient sera, to find auto-antibodies against targets related to disease, in e.g. autoimmune diseases or diseases where an auto-immune component is suspected.

- **Antibody Epitope Mapping:** Arrayed proteins are incubated with monoclonal or purified antibodies (provided by customer). Positive signals can be used to determine which epitope is recognized by the antibody. This approach is valuable for all clinicians and researchers who would like to work with antibodies that are not yet fully characterized, especially for developing new antibodies for diagnostics or treatments.
Methods

Sample Preparation

Different probes can be used for incubation of the Protein Arrays in screening experiments:

- undiluted serum samples (e.g. human, mouse, etc.) at a min. volume of 1000µl/screen
- purified antibody at min. volume of 250 µl (concentration of ~ 1 mg/ml) per screen
- hybridoma cell line supernatant at a min. volume of 1000µl per screen
- labelled peptides (biotin-, FITC-, or FAM-labelled) at a min. volume of 250 µl (concentration of ~ 1 mg/ml) per screen

Flow Chart for the Process of Serum Screening or Antibody Epitope Mapping

1. Incubation of imaGenes Protein Array with human serum (or other body fluids such as plasma, CSF) or the antibody to be characterized
2. Incubation with 2nd antibody linked to an appropriate detection agent (e.g. alkaline phosphatase, horseradish peroxidase)
3. Incubation with corresponding substrate (e.g. Attophos, ECL) and image detection (e.g. scanning, CCD camera, X-ray film)
4. Analysis of resulting images (manual scoring or computer assisted analysis using software packages)
5. Further validation of the results by means of the expression clones represented on the array

Protocols

The following protocol provides general advice for the handling of imaGenes Protein Macroarrays, the recommended processing for epitope mapping of antibodies and their utilization in screenings of sera for auto-antibodies.

General Advice

Never touch imaGenes Protein Arrays with unprotected fingers. When handling Protein Arrays wear gloves and apply flat forceps. Be aware that the surface of imaGenes Protein Arrays can be easily damaged. Thus, we recommend gripping imaGenes Protein Arrays only at their edges outside the spotting area with flat forceps. After incubation, air dry imaGenes Protein Arrays on Whatman paper. Store imaGenes Protein Arrays dry, at room temperature, between two sheets of Whatman paper.

Technical duplicates of all screening procedures are highly recommended!
Reagents and Instrumentation to be Supplied by User

General advice: in order to prepare solutions, please use ultra-pure H$_2$O where applicable.

Detection of His-tagged proteins (quality control) or of interacting proteins in a screening experiment

<table>
<thead>
<tr>
<th>1st Antibody</th>
<th>2nd Antibody</th>
<th>Supplier 1</th>
<th>Supplier 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGS-His-Antibody BSA free (100)</td>
<td>anti-mouse IgG (Fc-specific),</td>
<td>Qiagen (# 34650)</td>
<td>Isotype: mouse IgG</td>
</tr>
<tr>
<td>2nd Antibody</td>
<td></td>
<td>Amersham/GE (RPN 5781)</td>
<td></td>
</tr>
<tr>
<td>Alkaline Phosphatase conjugate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd Antibody</td>
<td>anti-human IgG (Fc-specific),</td>
<td>Sigma (A9544)</td>
<td></td>
</tr>
<tr>
<td>Alkaline Phosphatase conjugate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd Antibody</td>
<td>anti-human IgA (α-chain specific),</td>
<td>Sigma (A9669)</td>
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<tr>
<td>Alkaline Phosphatase conjugate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AttoPhos</td>
<td></td>
<td>Roche (11681982001)</td>
<td></td>
</tr>
</tbody>
</table>

Blocking

| Blocking solution | 3% Low fat milk powder in TBS-T |

Incubation

| TBS | 150 mM NaCl |
|     | 10 mM Tris-HCl, pH 7.5 |
| TBS-T (TBS + Tween) | 500 mM NaCl |
| | 20 mM Tris-HCl, pH 7.5 |
| | 0.05% Tween 20 |
| TBS-T-T (TBS + Tween + Triton X-100) | 500 mM NaCl |
| | 20 mM Tris-HCl, pH 7.5 |
| | 0.05% Tween 20 |
| | 0.5% Triton X-100 |

Scanning/Imaging

| Scanner, e.g. Storm 860 or FujiFLA3000 (Blue laser necessary!) | Amersham/GE Raytest |
Protocol: Screening of Human Serum for Auto-Antibodies / Epitope Mapping

Day One: Incubation with Primary Antibody

1. Place a single imaGenes Protein Array in a plastic box and rinse in 70% ethanol at room temperature.
2. Discard ethanol and rinse imaGenes Protein Arrays 2x (~2min) with ultra-pure H₂O in order to remove traces of ethanol.
3. Cover the bottom of another plastic box with 25 ml TBST-T, transfer the imaGenes Protein Array into this plastic box.
4. Carefully wipe-off all excess colony material from the imaGenes Protein Array using paper tissue (e.g. KimWipe). Proteins are now covalently bound to the imaGenes Protein Array. Hence, from this stage it is possible to handle two imaGenes Protein Arrays in a single box (if applicable, put the two arrays back to back, i.e. protein-binding surface facing the outside).
5. Wash imaGenes Protein Arrays shaking them 3x for 10 min at room temperature in TBST-T (we recommend a Rocky shaker for all shaking steps).
6. Rinse imaGenes Protein Arrays 2x briefly at room temperature in TBS in order to remove traces of TBST-T.
7. Wash imaGenes Protein Arrays shaking them 1x 10 min at room temperature in TBS.
8. Agitate imaGenes Protein Arrays for 2 h at room temperature in blocking solution.
9. Add the human serum / the antibody to be characterized to blocking solution:
   Alternative 1: If using bags, make sure three sides are sealed.
   – Place a single imaGenes Protein Array per bag only.
   – Add 10 ml blocking solution.
   – Add antiserum to be characterized, 1:50 – 1:100 / Add antibody to be characterized, 1:1000.
   – Seal last side, avoid air bubbles.
   Alternative 2: If using a plastic box, choose one with base dimensions close to those of the imaGenes Protein Array.
   – Place max. two imaGenes Protein Arrays per box; put the two arrays back to back, i.e. protein-binding surface facing the outside.
   – Add 25-50 ml blocking solution.
   – Add antiserum to be characterized, 1:50 – 1:100 / Add antibody to be characterized, 1:1000.
   – Seal box with tight-fitting lid.
10. Agitate imaGenes Protein Array overnight at 4 °C.

Day Two: Incubation with Secondary Antibody

11. Discard solution, place imaGenes Protein Array in a plastic box.
12. Wash imaGenes Protein Array 3x 15 min at room temperature in TBST.
13. Add 2nd antibody to Blocking solution:
   Alternative 1: Place 1 imaGenes Protein Array into bag.
   – Make sure three sides are sealed.
   – Add 10 ml blocking solution.
   – Add antibody: Anti-human IgG 1:5000 (or an alternative suitable antibody dependent on the Immunoglobulin isotype you would like to detect, e.g. Anti-human IgA / Anti-mouse or anti-rabbit IgG 1:5000).
   – Seal last side, avoid air bubbles.
   Alternative 2: If using plastic box, choose one with base dimensions close to those of the imaGenes Protein Array.
   – Place max. two imaGenes Protein Arrays per box; Protein Array “one” and Protein Array “two” back to back.
   – Add 50 ml blocking solution.
   – Add antibody: Anti-human IgG 1:5000.
   – Seal box with tight-fitting lid.
14. Agitate imaGenes Protein Array for 2 h at room temperature.
15. Wash imaGenes Protein Array 2x 10 min in 25 ml TBST.
16. Wash imaGenes Protein Array 2x 10 min in 25 ml TBS.
17. Wash imaGenes Protein Array 10 min in 25 ml AttoPhos buffer.
18. Drop 8 ml AttoPhos substrate on a Saran Wrap surface, overlay with imaGenes Protein Array (protein side down). Ensure that substrate is evenly distributed.
19. Place imaGenes Protein Array on glass plate of scanner (protein side down). Scan imaGenes Protein Array at 450 nm.
20. Analysis of resulting images (manual scoring or computer assisted analysis using software packages).

Detection of Interaction Signals on imaGenes Protein Arrays

Fluorescence labeling and detection methods are routinely used in the imaGenes Screening Service laboratories. In general we apply 2nd antibodies linked to alkaline phosphatase for incubation with AttoPhos (Roche) as highly sensitive fluorimetric substrate. Images are recorded by scanning (Storm 860, Amersham). It is also possible to illuminate the filters with longwave UV light (460 nm) and to take images using a high resolution CCD camera (e.g. Fuji LAS 3000) (Murphy et al., 2010).
Chemiluminescence detection is rather limited in performance due to signal instability, but the detection method was successfully used by some of our customers (Cepok et al., 2005; Holt et al., 2000), for example the Amersham ECL-Plus™ Detection System (ECL = Enhanced ChemiLuminescence). In this case a secondary antibody conjugated to horseradish peroxidase is incubated with luminol which gains an excited state following the oxidation in the presence of chemical enhancers. Images can be developed by scanning, by means of a CCD camera or even using a blue-light sensitive autoradiographic film (Mahlknecht et al., 2001).
Finally a HRP-coupled 2nd antibody was used together with precipitating tetramethylbenzidine-blotting (TMB-blotting) substrate (Pierce) to detect positive signals as blue spots directly on the filter (Cepok et al., 2005).
If antibodies are not available, radiolabeled protein (32P, 35S, or 125I) may be used for protein–protein interaction screening analyses as an alternative. In most cases, radioactive labeling requires the protein to be purified first, followed by direct radiolabeling of either tyrosine residues with 125I, or if an extrinsic phosphorylation site is present (which may intentionally be cloned into the protein), with 32P in conjunction with a commercially available kinase (Pless et al., 2008).
An alternative to radiolabeling is to biotinylate purified protein probes and then detect them using e.g. streptavidin conjugated to alkaline phosphatase or to HRP (Kowenz-Leutz et al., 2010).

Guidelines for Analysis of Macroarray Results

![Fig. 3](image.png)

**Fig. 3** Serum screening of imaGenes Protein Arrays. The Protein Array was overlaid with patient serum containing auto-antibodies against human proteins. Reacting proteins were detected via AP-conjugated anti-human secondary antibodies and AttoPhos substrate. Images were obtained by scanning. Identification of positive clones was carried out with the AIDA software.
Figure 3 shows a representative result for any type of protein-protein interaction analysis using imaGenes Protein Arrays. Depending on the macroarray type (hEx select or UniPEx) the proteins are arranged in “5x5” or “3x3 blocks”. All proteins are arrayed twice so that they appear as duplicate spots in a particular pattern within a block after successful hybridization. These technical duplicates allow a reliable identification of positive signals even in case of an irregular background.

The resulting images can be easily analyzed manually applying the imaGenes Scoring Template indicating the corresponding spotting pattern. The positions (x,y coordinates) of positive signals assigned in this way can be easily related to a particular clone via the also delivered annotation table (see Fig.4). The annotation table furthermore comprises additional information to each protein (e.g. sequence information, gene symbol and description) allowing for a rapid identification of binding interactions and facilitating a biological interpretation of the results (e.g. by performing a pathway analysis).

![Fig.4 Excerpt of an annotation table of the proteins represented on the array](image)

Software packages such as AIDA (Raytest) permit a convenient computer-assisted analysis of digital images. The grid describing the protein/clone positions on the imaGenes Protein Array as well as the annotation table can be loaded into the software, so that all available information concerning a protein are displayed during the analysis process and in the resulting probe table.

![Fig.5 Screenshot of an incubation image visualized and analyzed with AIDA image analysis software (Raytest) that was adjusted to the imaGenes Protein Array format. After aligning the spots, single proteins can be conveniently identified, as the annotation table can be loaded into the software. The array result table shows the intensity of the spots and background values. One protein is always represented by two spots in a particular pattern within the same block (all proteins are spotted in duplicate). Positive protein spots are highlighted on the grid in the image, on the schematic display of the array as well as in the result table below.](image)
Further Validation of Identified Protein-Protein Interactions

One clear advantage of imaGenes Protein Arrays is that expression clones identified as "positives" during a screening procedure can readily be retrieved from imaGenes. All clones can be ordered via imaGenes online web portal GenomeCube® by searching for the Clone IDs indicated in the annotation table. Thus, large quantities of the recombinant protein can be produced for further experimental analyses. His-tagged recombinant human proteins can be purified using chelate-affinity chromatography. For protein expression protocols please refer to the respected manual from Qiagen concerning the bacterial expression vector series pQE (The QIAexpressionist™ – A handbook for high-level expression and purification of 6xHis-tagged proteins). The protein expression cDNA libraries represented on imaGenes Protein Arrays were even used for protein expression and purification in a high throughput format using 384 microtiter plates (Büssow et al., 2004, 2000, 1998).

The proteins expressed and purified from the corresponding clones detected in a screening experiment can be used for the further validation and specification of the discovered interactions. For example after the identification of the molecular targets from a novel antibody the specificity and sensitivity of the interactions can be confirmed by Western immunoblotting and transfectant studies (Kijanka et al., 2009; Larkin et al., 2004). If the proteins were expressed and purified in a large scale format, specific protein microarrays can be produced (Horn et al., 2006).

Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Suggestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>No incubation signals</td>
<td>▶ Check quality of serum in a conventional Western-blot experiment</td>
</tr>
<tr>
<td></td>
<td>▶ Apply serum at a higher concentration</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Too many incubation signals (many more than expected)</td>
<td>▶ Check quality of serum in a conventional Western-blot experiment</td>
</tr>
<tr>
<td></td>
<td>▶ Apply serum at a lower concentration</td>
</tr>
<tr>
<td></td>
<td>▶ Repeat pre-absorption step (onto E. coli proteins)</td>
</tr>
<tr>
<td></td>
<td>▶ Wash more stringently (TBST-T or use higher Tween concentrations)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>High (fluorescent) background</td>
<td>▶ Use other blocking solutions (FCS, fish-gelatin, or higher concentrated milk powder); wash more stringently (e.g. with higher Tween concentrations)</td>
</tr>
</tbody>
</table>
References

2010

Protein interactions with the platelet integrin alpha(IIb) regulatory motif.

Protein macroarray profiling of serum autoantibodies in pseudoexfoliation glaucoma

Constitutively overexpressed 21 kDa protein in Hodgkin lymphoma and aggressive non-Hodgkin lymphomas identified as cytochrome B5b (CYB5B).

Diagnostic and prognostic biomarker discovery strategies for autoimmune disorders.

Integrated Protein Array screening and high throughput validation of 70 novel neural calmodulin binding proteins.

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