Comparing the sensitivity of engine protein arrays with a commercially available ELISA for the detection of TRIM21

Introduction

The study of protein-protein interactions is of great importance for biological and medical research. Several approaches to map interactions between different proteins have been proposed and implemented, but suffer from individual drawbacks, including high costs and the need for large sample sizes [1]. A particularly interesting method to study these protein-protein interactions is the use of protein arrays [2].

Protein arrays have been used since the early 1980s by immobilizing antibodies on a solid surface [3]. However, the first arrays produced in this way had a relatively small diversity of bound antibodies and were not suitable for mass application. Since then, the technology of arrays has been greatly improved and benefited from the concurrent development of DNA microarrays, which operate on similar principles to detect DNA-DNA or DNA-RNA interactions.

Protein arrays rely on the immobilization of various proteins on test patches (spots) on the surface of a solid chip. When a sample is incubated with the array, proteins from the solution can bind to specific spots, while unbound proteins are washed away. Spots with bound proteins can be identified using functionalized antibodies specific for the proteins in the test solution (which can sometimes also be antibodies themselves). The presence of bound antibodies, by coupling to an enzymatic process, produces an optical signal that can be detected with a colorimetric instrument [4].

Protein arrays have several advantageous properties. For example, protein arrays not only allow the study of other proteins (protein-protein, protein-peptide or protein-antibody interactions) but can also detect interactions of proteins with DNA or RNA (protein-nucleic acid interactions) [4].

Protein arrays also allow the simultaneous analysis of more than 15,000 different interactions, depending on the density of the different spots on an array. Customization of the chip to the specific requirements of the array is also possible and allows great flexibility of the method.

engine protein arrays

engine offers a selection of protein arrays for the analysis of auto-antibody profiles of complex sera, cross-reactivity of monoclonal antibodies and other types of protein-protein interactions. On each of these arrays, several thousand different proteins are expressed and immobilized directly on polyvinylidene fluoride (PVDF) membranes (Figure 1). Clones are arranged in multiple copies in distinct patterns to avoid false positive results (Figure 2 and Figure 3).

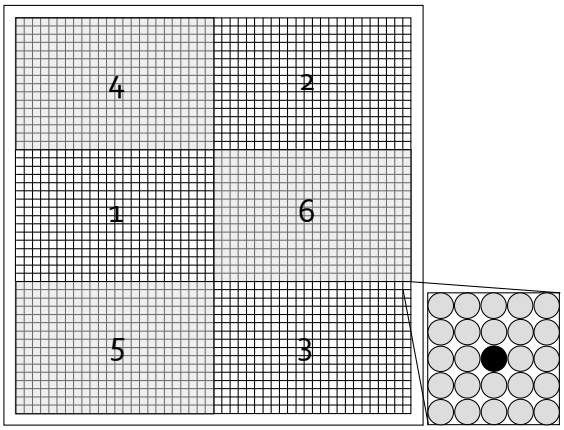

Figure 1: Schematic view of an engine protein array, printed in 5x5 pattern. Numbers 1-6 indicate the fields, clones from 384 well plates are spotted into. Clones are spotted there in distinct patterns (see Figure 2 and Figure 3) in duplicate, serving as a build-in positive control, since spots are only considered positive, if both spots can be detected. Pattern 1 is spotted in fields 1-6 from the first 6 384 well plates. Pattern 2 starts from plate 7 in field 1 and so on. By doing so, up to > 27.000 clones can be placed on the array in duplicate. Zoomed out part shows one block of the array. In 5x5 patterns, one block consists of up to 12 clones in duplicate and one central guiding dot.

cDNA collection

To construct the expression libraries for engine protein arrays, cDNA was prepared from either human fetal brain, colon and lung tissue and T-cells, using poly(A)+ RNA by oligo(dT) priming. The products were directionally cloned into modified vectors for IPTG-inducible expression of His_6-tagged fusion proteins and transformed into either *E. coli* SCS1 cells (for human fetal brain origin) or *E. coli* DH10B cells (for colon and lung tissue and T-cells). After selection of the desired clones by checking for expression, they were rearrayed into 384-well microtiter plates and *N*-terminally sequenced for verification of the cloned gene.

Spotting of expression libraries for engine protein arrays

To produce engine protein arrays, PVDF membranes are activated with ethanol, washed twice with water, and incubated with LB-medium. Then, the membranes are positioned on pre-soaked Whatman paper and transferred to the spotting robot. There, the clones arranged in 384-well microtiter plates, are spotted onto the membrane using a spotting device with 384 needles.

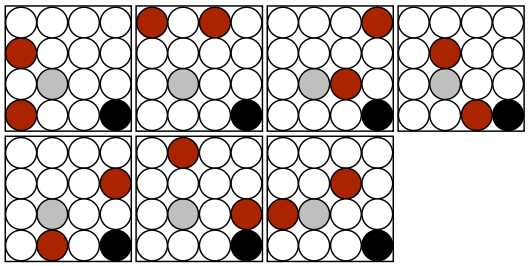

Figure 2: Patterns 1-7 (from left to right, highlighted in red) in which clones are spotted in duplicate in a 4x4 pattern on engine protein arrays. All patterns are spotted in each block. For better visibility, they are shown in separate blocks and highlighted in red. Since there is no middle spot available serving as the guiding dot for the block, the bottom right was chosen for this purpose (marked in black). The corresponding spot to this pattern is left without an E. coli clone or dye and can be used as background for the individual block (marked in grey).

For UniPEx protein arrays, clones are spotted in a defined duplicate pattern within spotting squares that consist of up to seven duplicate clones (providing internal controls to eliminate false positives) and one reference spot per square to facilitate membrane alignment (4x4 pattern, Figure 2).

For hEXselect protein arrays, clones are spotted in a defined duplicate pattern within spotting squares that consist of up to 12 duplicate clones and one central reference spot per square (5x5 pattern, Figure 3).

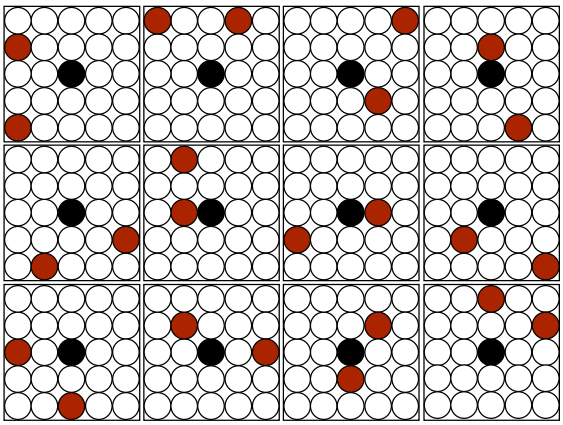

Figure 3: Patterns 1-12 (from left to right, highlighted in red) in which clones are spotted in duplicate in a 5x5 pattern on engine protein arrays. The black centre spot marks the guiding dot for the block. All patterns are spotted in each block. For better visibility, they are shown here in separate blocks and highlighted in red.

Protein library expression and protein array production

For expression, bacterial clones on the membrane are incubated overnight on 2×YT-agar plates containing 2 % glucose and 100 µg/ml ampicillin at 37 °C and induced for protein expression for 3 h at 37 °C on agar plates containing 1 mM IPTG. These protein filters are denatured on pre-soaked blotting paper in denaturation buffer* for 10 min, neutralized for 2× 5 min with neutralization buffer* and incubated for 15 min in NaPi buffer*. All proteins on the arrays are verified for expression by detecting the His_6-Tag using an anti-His antibody RGS·His (Qiagen), 1 array per spotting run.

*Denaturation buffer – 0.5 M NaOH, 1.5 M NaCl
*Neutralization buffer – 1 M Tris–HCl, pH 7.5, 1.5 M NaCl
*NaPi buffer - 0.05 M di-sodium dihydrogen-phosphate, pH 7.2

engine protein array portfolio

UniPEx protein arrays are normalized bacterial protein expression libraries that cover

approximately one-third of the human proteome. Here, recombinant proteins are expressed from cDNAs derived from human fetal brain, lung, colon, and T lymphocytes. They contain over 15,000 sequenced, annotated and verified cDNA clones that represent over 6,000 unique proteins. Clones were selected to be in frame, so these arrays present only a small amount of neoantigens. Protein products represent mostly full-length proteins, poly- and oligopeptides.

hEXselect protein arrays consist of a bacterial protein expression library containing >24,000 cDNA clones, covering over 7,000 unique human proteins. Recombinant proteins are expressed from cDNAs derived from human fetal brain tissue that were cloned into an expression vector, with an additional *N*-terminal His_6-Tag. Expressed protein products represent full-length proteins, oligopeptides, polypeptides and neoantigens.

If a pre-screen has been performed on either hEXselect or UniPEx protein arrays, it can be useful to assemble specific and individual subsets of 500 – 3900 clones of interest in duplicate to screen larger cohorts of patient samples. In this way, a larger number of samples can be screened for suitable biomarkers more quickly and cost-effectively.

ELISA

Another commonly used method to study known protein interactions is the Enzyme-linked Immunosorbent Assay (ELISA), which works on a similar principle to protein arrays but has the disadvantage that only one interaction is studied at a time.

In addition, it is not possible to screen samples for unknown biomarkers, as ELISA only present a known protein in its setting.

Since ELISA are commonly used for diagnostic purposes, the question arises whether engine protein arrays have comparable sensitivity and specificity to ELISA and whether the interactions found with engine protein arrays can be reproduced in an ELISA screen and vice versa. Therefore, engine performed a series of experiments to investigate how their hEXselect and UniPEx arrays compare to a commercial ELISA for the antibody effector protein Tripartite motif-containing protein 21 also known as TRIM21.

TRIM21

TRIM21 is known to interact with autoantigens in patients with systemic lupus erythematosus and Sjögren's syndrome. A test for TRIM21 antibodies supports the differential diagnosis and the clinical monitoring of systemic rheumatic inflammatory autoimmune diseases.

Materials and Methods

ELISA

The ELISA (ORGENTEC Anti-SS-A 52, Order number ORG 652) was performed according to manufacturer specifications [5]. 100 µl of diluted samples, controls and blanks were pipetted into microtiter plates, incubated for 30 minutes, and washed 3 times. Afterwards 100 µl enzyme solution was added, incubated for 15 minutes, and washed 3 times. The enzyme substrate was added onto the plates and again incubated for 15 minutes. Finally, stopping solution was added followed by 5 minutes incubation time and consecutive readout of light absorption at 450 nm. The concentration of TRIM21 antibodies in each sample was calculated by comparison with a standard curve generated by the control samples with a 4-parameter curve-fit. 4 samples of patient sera (provided by in.vent Diagnostica GmbH) were analysed in dilutions ranging from 1:100 – 1:25,600. High concentration samples were diluted to match the dynamic measuring range of the assay. Each sample was measured twice to increase result accuracy. Control samples were used without further dilution.

Protein array probing

Incubation with primary antibody

Experiments were performed with three types of arrays, the first hEXselect (order no 1003), and the second UniPEx (order no 1008). The third type of array was a subarray (order no 1009) with > 1,000 proteins spotted in duplicates. All arrays were treated the same, the only difference was, that for

array 1009, lesser incubation volumes were used (5 ml instead of 50 ml).

The protein arrays were first activated with ethanol and washed with demineralized water (2x) before excess colony material was wiped off the surface of the array. After washing once with water, the protein arrays were agitated in blocking solution for 2 hours*. Diluted serum (provided by in.vent Diagnostica GmbH) was added, and the arrays were shaken overnight at room temperature. Dilutions of the samples were chosen according to the ELISA experiments (data not shown). After being able to detect antibodies against TRIM21 in 3 samples at a dilution of 1:25,600, further experiments were carried out.

In the second experiment, samples were further diluted to obtain a total enzyme activity of 0.78 U anti TRIM21 antibody and 0.0078 U, respectively, to determine the lower end of microarray sensitivity, as the concentration used in ELISA assays proofed to be too high. For 78 U anti TRIM21 antibody, 50 µl of the serum with 1555 U/ml were diluted in 50 ml buffer. For 0.78 and 0.0078 U total, the volume of the serum corresponds to 0.5 and 0.005 µl in 50 ml buffer, respectively.

For experiments with array 1009, the serum (170622) was diluted to 0.0015 – 3 U anti TRIM21 (calculated from ELISA experiments) in 5 ml buffer. The negative serum (241404) was used with 10 µl serum in 5 ml. This corresponds to 0.01 U (calculated from ELISA results).

Incubation with secondary antibody

The previous solution was discarded, and the array was washed with wash buffer* at RT for 3 x 10 minutes. 50 ml of blocking solution and the anti-human IgG-AP antibody (Seramun, A-002-1-AP) was added at a dilution of 1:20,000 (2.5 µl of antibody in 50 ml/0.25 µl antibody in 5 ml), then the arrays were shaken for 2 hours. The arrays were then washed with washing buffer (2 x 10 min), TBS (2 x 10 min), and APE buffer* (1 x 10 min). 10 ml or 1 ml (for array 1009) of SeramunPurple® prec substrate (Seramun, S-008-5-BCIP) was pipetted on the array and incubated for 5-10 min. The reaction was stopped by transferring the arrays into demineralized water. The arrays were then placed protein side down on an EPSON Expression 12000XL scanner and scanned.

*Blocking solution – Candor BSA block
*Washing buffer – Candor wash buffer
*APE buffer - 1 mM $MgCl_2$, 100 mM Tris-HCl, pH 9,5

Analysis

The tiff images were acquired by scanning with an Expression 12000XL scanner from Epson, using an optical resolution of 800 ppi. Analysis was performed using VisualPattern20, an analysis program, specifically designed to analyse engine protein arrays. Hits were considered positive only if both spots were clearly positive.

Results

ELISA

Using the standard sample solutions provided, we were able to create a 4-parameter curve fit for enzyme activity against medium optical density (see Figure 4 A).

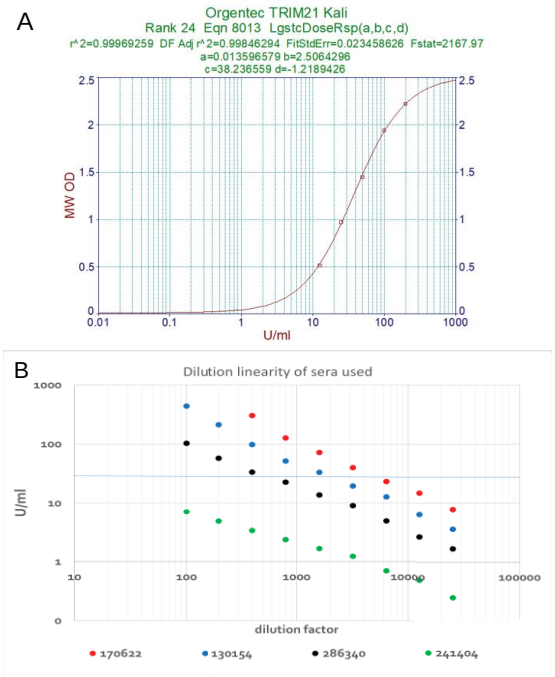

Figure 4: (A) 4-parameter curve fit for TRIM21 antibody detection (ELISA), calculated from results of the calibrator measurements. (B) Dilution linearity of sera used in ELISA. The blue line indicates 25 U/ml – the cut-off value established for ELISA. Sera showing less than 25 U/ml are considered negative for TRIM21 antibodies, as reported in the manufacturer's manual [5].

Sera with activities >25 U/ml were considered positive for TRIM21 antibodies, otherwise the sample was considered negative as described in the ELISA manual.

For the 4 sera tested, the following concentrations of anti-TRIM21 antibody in the respective sere were (Figure 4 B) calculated from the ELISA standard curve:

Serum 1 (170622): 1555 U/ml - positive
Serum 2 (130154): 645 U/ml - positive
Serum 3 (286340): 212 U/ml - positive
Serum 4 (241404): 10 U/ml - negative

Engine Protein microarray

Analysis of the protein array showed clearly positive spots for TRIM21 antibodies for the three sera that also tested positive in ELISA (data for serum with 1555 U/ml (170622), see Figure 5-Figure 7, other data not shown), while no signal was detected in the case of the ELISA-negative serum (241404) (Figure 8).

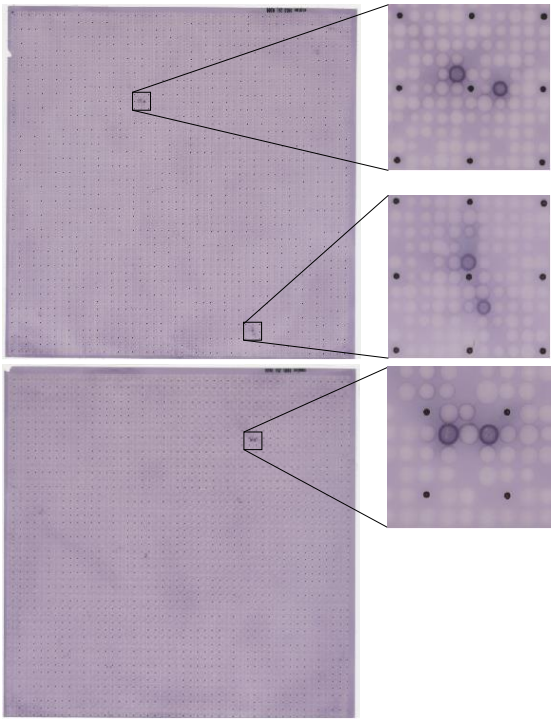

Figure 5: Serum 170622 was diluted to a total of 77.5 U anti-TRIM21 antibody (calculated from ELISA results). Arrays were incubated with serum and an anti-human IgG antibody (AP coupled). Positive hits were detected using SeramunPurple® prec, (NBT/BCIP, Seramun). Positive hits are clearly detectable on both, hEXselect (upper panel, pattern 10 and pattern 4, see Figure 3) and UniPEx (lower panel, pattern 2, see Figure 2) protein arrays.

This was observed with both hEXselect and UniPEx protein arrays. For better visibility, the detected hits were zoomed out.

TRIM21 is located in 2 different areas of the hEXselect array (pattern 10 and pattern 4, see Figure 3 and zoomed regions of upper panels of Figure 5-Figure 7), while only 1 clone is present on the UniPEx array (pattern 2, see Figure 2 and zoomed regions of lower panels of Figure 5-Figure 7).

Anti-TRIM21 antibodies could be detected for all dilutions including down to a total of 0.0078 U anti TRIM21 antibodies (Figure 7). The units used for incubation of hEXselect and UniPEx arrays, were calculated from ELISA experiments. Further experiments showed that TRIM21 antibodies could be detected even at a total of 0.0015 U on the array (Figure 9).

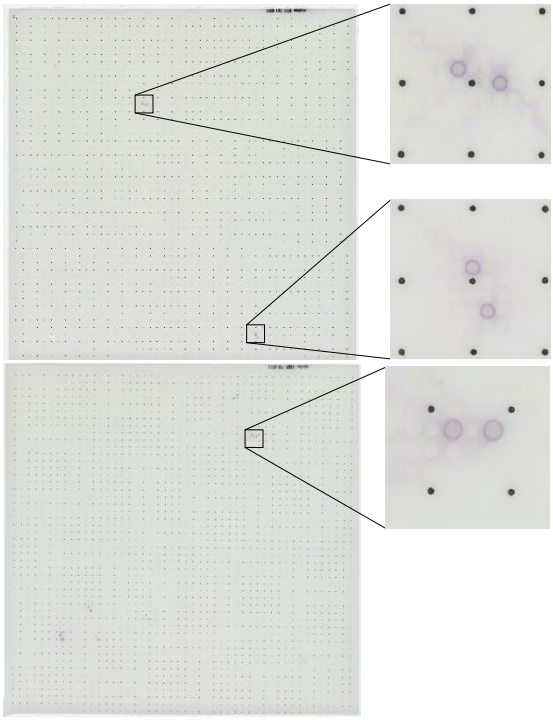

Figure 6: Serum 170622 was diluted to a total of 0.78 U anti-TRIM21 antibody (calculated from ELISA results). Arrays were incubated with serum and an anti-human IgG antibody (AP coupled). Positive hits were detected using SeramunPurple® prec, (NBT/BCIP, Seramun). Positive hits are clearly detectable on both, hEXselect (upper panel, pattern 10 and pattern 4, see Figure 3) and UniPEx (lower panel, pattern 2, see Figure 2) protein arrays.

In addition, we observed that dilution of sera decreases the background staining of arrays dramatically and therefore can increase the

quality of the results under certain circumstances. More concentrated samples can produce a serum-specific background due to the high overall protein concentration in serum, while TRIM21-specific spots are oversaturated. Reducing the serum-specific background by dilution resulted in only a slight decrease in absorbance at the TRIM21 spots, increasing contrast (see Figure 6).

In case of the negative serum (241404) (Figure 8), one could argue that the background staining could be too dark to detect 0.01 U on the array. The staining of the arrays appears to be additive, therefore, it is highly likely that even a weak signal could be detected – such as for pattern 4 (see Figure 3) in the same block. This signal is quite weak but can be detected and is listed in the corresponding result file (data not shown).

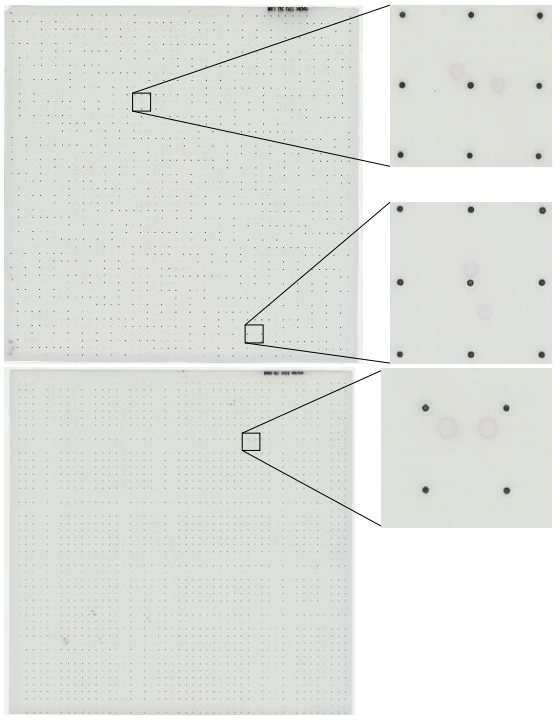

Figure 7: Serum 170622 was diluted to a total of 0.0078 U anti-TRIM21 antibody (calculated from ELISA results). Arrays were incubated with serum and an anti-human IgG antibody (AP coupled). Positive hits were detected using SeramunPurple® prec, (NBT/BCIP, Seramun). Positive hits are clearly detectable on both, hEXselect (upper panel, pattern 10 and pattern 4, see Figure 3) and UniPEx (lower panel, pattern 2, see Figure 2) protein arrays.

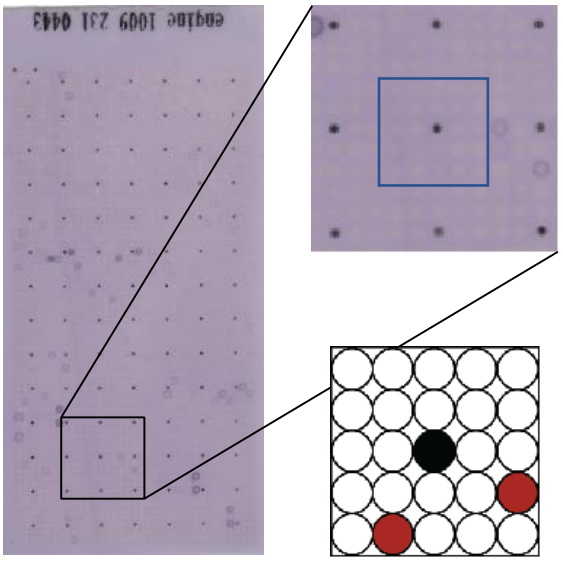

Figure 8: 10 µl (corresponding to 0.01 U total, calculated from ELISA results) serum (241404) in blocking buffer were incubated on the array, followed by incubation with an anti-human IgG antibody (AP coupled). Positive hits were detected using SeramunPurple® prec, (NBT/BCIP, Seramun). With 10 µl serum, many positive hits could be detected, but not for TRIM21. The block, where TRIM21 is expressed, was zoomed out for better visibility (right upper panel, blue square), but TRIM21 could not be detected (pattern in which TRIM21 would appear was included on lower panel for better visualization).

Comparison of results

While ELISA results can be given in U/ml, this is not possible in the same way for engine protein arrays.

For the arrays, the total antibody activity in the serum (U) used must be considered and not the volume specific antibody activity (U/ml). For example, if 1 U of antibody is present in 1 µl, then this 1 µl can be diluted in 25 ml or in 50 ml and used to incubate the array – both volumes are possible for incubation. The total amount (U) used will then remain the same, and the same intensity of interaction will be detected whether the 1 µl was diluted in 25 or 50 ml. However, if simply converted to U/ml, the result would be different as there would be more U/ml in 25 ml as less volume would be used. Conversely, if the dilution were set to have 1 U in 50 ml, then only 0.5 U would be incubated if 25 ml of this dilution were used, although the U/ml would be the same.

For the ELISA, the functional assay sensitivity is 0.5 U/ml. To compare this to the sensitivity of the arrays (0.0015 – 0.0078 U total), we recalculated

the sensitivity of the ELISA to 0.05 U in total per well, since the assay volume is 100 µl per reaction in the well. This shows that engine protein arrays show a superior functional assay sensitivity for the detection of anti TRIM21 antibodies than the commercially available ELISA.

Furthermore, it is possible to detect other potential biomarkers and protein interactions on the same membrane.

Summary

We found that engine protein arrays have a higher sensitivity for the detection of TRIM21 than the tested commercial ELISA. The functional assay sensitivity of the ELISA is 0.5 U/ml (calculated to be 0.05 U in total per well), while hEXselect and UniPEx were determined to have a sensitivity of 0.0015 - 0.0078 U to anti TRIM21 antibodies in total. This result shows a clear advantage for engine protein arrays compared to ELISA.

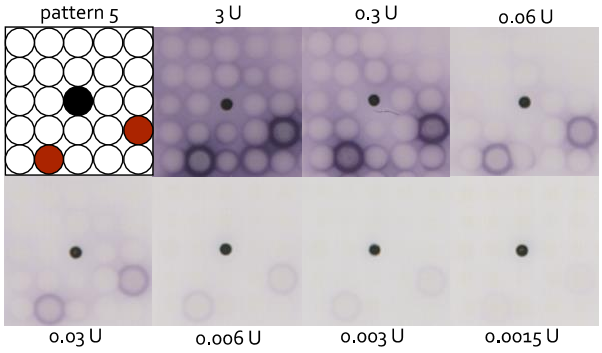

Figure 9: Cropped out blocks of subarrays (1009, for whole array see Figure 8) incubated with different amounts of Serum 170622. The blocks show the clone expressing TRIM21 in pattern 5. Arrays were incubated with calculated U of anti-TRIM21 from 0.0015-3 U in total.

An additional advantage is that the protein array also appears to have better specificity, as there is a clearer distinction between positive and negative sera. With the ELISA, there is an arbitrarily measured activity (25 U/ml) below which serum is considered negative for TRIM21 antibodies (Figure 4 and [5]). With engine protein arrays, the presence of antibodies could be detected at much lower concentrations, down to 0.0078 U. Furthermore, the serum, which tested negative for TRIM21 in the ELISA, showed no binding to the TRIM21 spot on the array.

In total, the higher sensitivity and specificity of engine protein arrays paired with the parallel testing capabilities of > 15,000 proteins and peptides in one experiment indicate a clear benefit for them to be used in clinical and research settings. Protein arrays would not only reduce the number of false positives and false negatives, but also facilitate the search for new biomarkers in the same experiment, while ELISA is mainly used to confirm existing hypotheses.

Protein arrays are powerful tools for the discovery and validation of biomarkers. The sensitivity and specificity are comparable or even higher compared to ELISA and an undeniable advantage is the possibility, to discover more than one interaction in one experiment. Furthermore, by using precipitating substrate, interactions can be discovered by using a simple colour office scanner. No further expensive equipment is needed in this case.

But one must keep in mind, that the use of protein arrays is a screening technology presenting yes/no results. To establish concentration dependencies, ELISA measurements prove to be the method of choice.

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