A Luminex bead-array workflow enables efficient confirmation of antigenic proteins derived from *engine*'s protein array-screening.

Introduction

Proteins immobilized on planar surfaces (macro- and micro-arrays) allow identification of antibody reactivities to multiple antigens in parallel and therefore allow high throughout biomarker discovery. The identified candidate markers need to be further confirmed and validated using targeted assays. Bead-arrays provide an attractive alternative when proteins are immobilized on paramagnetic beads and sample processing can be conducted in an up to 500-multiplexed format using Luminex® bead-array technology.

engine the biomarker company (https://protein-arrays.bio/), provides the most comprehensive panel of human expression clones and protein array-based screening for identification of sero-reactive antigenic signatures, by presenting more than 15,000 proteins, peptides and neoantigens on one surface, to be analysed in a single experiment. All expression products are present in duplicate and represented in distinct patterns, serving as a built-in binding control, since hits are only considered to be positive if both spots show comparable intensities [1].

A complete immunomics ISO9001-qualified workflow combining different techniques and array formats has been implemented at AIT for screening, confirmation and validation of (auto)-antibody signatures (https://peppipe.ait.ac.at/; https://molecular-diagnostics.ait.ac.at/services/immunomics/) – see Figure 1. Here we show that antigenic proteins elucidated by *engine*'s protein array-based screening can be efficiently put forward by a high throughput-multiplex-bead-array format which is also suitable for clinical use.

In this work we present a comparison of protein array and Luminex bead-array analysis using expression clones showing antigenic reactivities on macro-arrays in sera from rheumatoid arthritis (RA) patients. Expression clones, selected from array experiments, were used for micro scaled recombinant protein expression and purification to then generate a 72-plexed Luminex bead-array (57 antigens, 3 vector controls "blank vectors", 12 control proteins) to confirm antigenic reactivities of candidate markers.

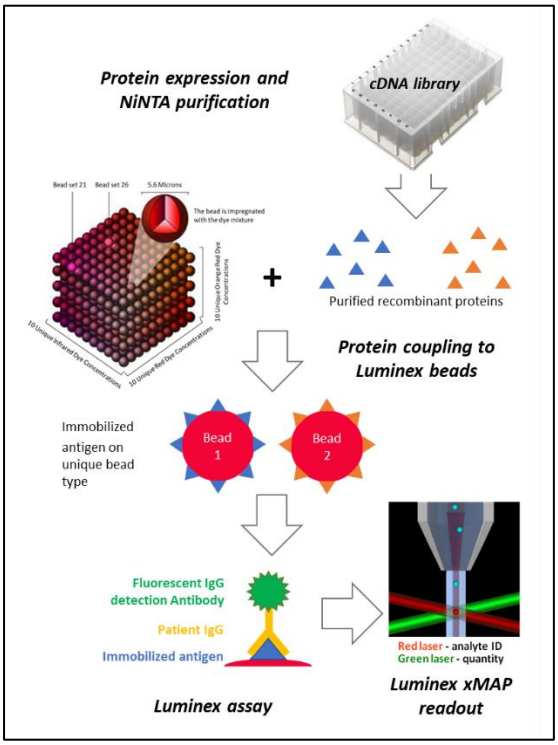

Figure 1: Schematic representation of the experimental workflow using a Luminex-bead-array

Experimental Design

Identification of candidate marker proteins

Sera from 8 RA patients (provided by in.vent Diagnostica GmbH) were tested on *engine* protein subarrays (819 clones, 21 controls in duplicate) for identification of autoantibodies. Arrays were re-hydrated, *E. coli* debris was removed from the surface and the membranes blocked for 2 hours in blocking solution. Patient serum was diluted in

blocking solution (10 µl in 2 ml) and incubated overnight. After 3 washing steps, arrays were incubated with an anti-human IgG-alkaline phosphatase (AP) conjugated antibody for 2 hours, washed again 4 times and interactions were visualized using Attophos® and a Storm 860 fluorescence scanner. All incubation steps were carried out at room temperature on a rocking surface. Positive interactions were evaluated and marked with intensity scores ranging from 1-3 (1-low, 2-medium, 3-high intensity) using VisualPattern20 – a software especially designed to evaluate *engine* protein arrays.

Expression and purification of recombinant proteins

Sero-reactive antigens from the described protein array-based screening were selected by *engine* and corresponding expression clones were provided for recombinant protein expression. The 57 *E. coli* clones containing human cDNA vectors from *engine*'s hEXselect and UniPEx library and 3 *E. coli* clones containing blank vectors were cultivated at 37°C in 2 ml TB-medium. Recombinant protein expression was induced by addition of IPTG. After enzymatic and mechanic (sonication) lysis, the His-tagged recombinant proteins were purified with Ni-NTA beads.

Immobilization on Luminex® magnet beads

Each purified protein was immobilized on a unique paramagnetic microsphere type (bead-type) via amine coupling. Upon protein coupling onto beads, the different bead types were stored until use. For sample processing bead-types were mixed to generate the bead-array [2].

Serum samples

Eight (n=8) serum samples from patients, diagnosed with RA, which had been analysed on *engine* protein arrays were provided for comparative bead-array analysis. IgG - purified from samples using Melon Gel and concentration standardised IgG (0.1mg/ml) - as well as serum-dilution (1:200) was used for bead-array analysis. An independent set of seropositive and seronegative RA samples (n=48) was analysed in parallel on the bead-array [3].

Multiplexed Luminex® bead-array analysis

Proteins immobilized on different color-coded fluorescently labelled bead-types were pooled to generate the bead-array. For comparing native and denaturated proteins, half of the bead pool was incubated 10 min with 2% SDS at 70°C (denaturating condition) immediately before adding samples for analysis. The other half was directly incubated with the same samples without pre-treatment (native condition). Both sera diluted 1:200 in assay buffer as well as purified IgG were analysed (Figure 2). After 1 h sample-incubation and washing the quantity of specifically bound human IgG antibodies was detected with 1:200 diluted "goat anti human-IgG"- phycoerythrin conjugated detection-antibody.

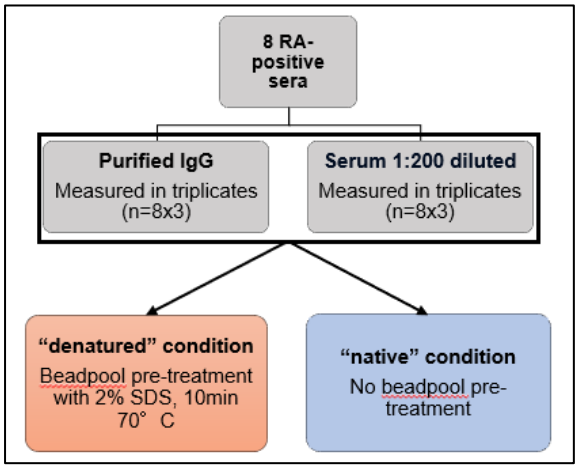

Figure 2: Schematic overview of tested samples and Luminex assay conditions. Denatured conditions refer to pre-treatment of the Luminex bead pool with 2% SDS for 10 min at 70°C prior to sample incubation while native condition refers to no pre-treatment of the bead-pool.

Data Acquisition and Data analysis

The raw MFI signals (median fluorescence intensity derived from the detection-antibody) for each sample and all different bead-types were determined with the Luminex® FlexMAP 3D system. A cut-off was defined by calculating the

mean MFI+2SD signals of "blank vector" measurements (beads coupled with NiNTA eluates from clones with the blank vector). A sample was classified as reactive to an immobilized antigen, when the measured MFI signal exceeded the defined cut-off value. The number of sero-reactive samples for each antigen was used for comparative analysis of the Luminex bead assay and the *engine* protein array platform and correlation coefficients (r) calculated.

Results

Results of array experiments

8 sera of RA patients were used for the discovery of potential RA-biomarkers on *engine* RA-protein arrays and analysed with VisualPattern20. Examples for positive hits of 2 different patients on *engine* arrays are shown in Figure 3. It is apparent, that RA patients share several markers but also display individual hits.

In total, 101 positive hits were identified for 8 RA patients, 26 of them for more than 1 person. Those reactants included full-length proteins (e.g., RPL18A), peptides (e.g., RPL21) and neoantigens (e.g., ANKS6). The 26 multi-reacting and additional 31 antigens were selected for the comparative analysis using Luminex® bead-arrays.

Interestingly, the neoantigen resulting from the ANKS6 gene shows 57% similarity to the Epstein-Barr nuclear antigen 1. Since more than 90 % of humans are infected with the Epstein-Barr-virus at one point in their live this sheds a light on the high binding frequency of 7 out of 8 positive reactions regarding this peptide (Figure 4).

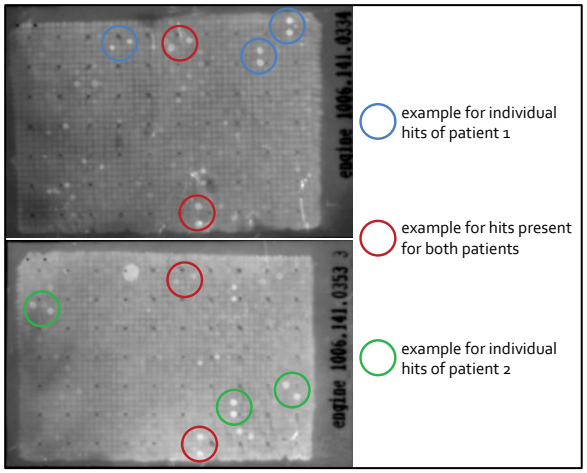

Figure 3: Display of 2 engine RA-subarrays probed with sera of 2 different RA-patients. Upper Array – patient 1, lower array patient 2. Hits for autoantibody-reactivity were visualized using an anti-human IgG-AP-antibody and Attophos®, scans of arrays were performed on a Storm 860 fluorescence scanner. Blue circles – example for hits only present for patient 1, green circles – example for hits only present for patient 2, red circles – example for hits present for both patients.

				Luminex assay ranking (# of reactive samples; n = 8x3)			
				purified IgG		Serumdilution (1:200)	
Clone	Gene	Content_384	Microarray ranking (# of reactive samples; n = 8)	native condition	denatured condition	native condition	denatured condition
ELIB1006p5G7	ANKS6	MPMGp800A24548	7	13	11	10	6
ELIB1006p4G3	RPL21	MPMGp800P13539	6	1	2	0	0
ELIB1006p12B3	ZNF358	MPMGp800L02597	4	8	21	15	18
ELIB1006p12F7	RPL18A	MPMGp800D13601	3	2	2	0	0
ELIB1006p1A6	CCDC174	MPMGp800A20506	3	0	1	3	2
ELIB1006p8A5	MAZ	MPMGp800M18568	3	5	11	21	22
ELIB1006p7E8	RPL18A	MPMGp800D11564	3	1	2	0	0
ELIB1006p6E6	NUMA1	MPMGp800G09554	2	2	8	0	2
ELIB1006p8A11	RPL18A	MPMGp800O19568	2	5	7	0	3
ELIB1006p10A11	ZNF358	MPMGp800B01582	2	2	21	1	9
Correlation coefficient of Microarray vs. Luminex ranking:				0.56	-0.07	0.25	-0.02
Correlation coefficient of Luminex "nativ" vs. "denatured" condition				0.75		0.90	

Figure 4: Summary table of antigenic IgG-reactivities obtained on macro-arrays and the targeted Luminex bead-array. Both platforms are compared via correlation coefficients (r) based on the number of immune-reactive samples exceeding the cut-off value.

Microscale protein expression and yield

Microscaled protein-expression from clones provided by *engine* obtained total protein yield ranging from 4-128 µg (mean=41 µg) for the 57 coding vectors, only 3 of which obtained less than 7 µg protein. Blank vectors yielded in 1.9-2.7 µg (mean=2.3 µg) total protein. This expression method resulted in sufficient protein amounts for a minimum of 860 Luminex reactions (at average 8000 reactions are feasible).

Raw Luminex bead-array data

For all 96 samples tested with the 72-plexed Luminex bead-array Median Fluorescence intensity (MFI) values were ranging from 1,304-460,789 (average=34,663) under native conditions and 1,744-454,937 (average=46,268) under denatured conditions. The 8 RA-positive samples provided by *engine* showed MFI values ranging from 1,304-205,759 (average=13,821) applying a 1:200 serum dilution and 4,621-460,789 (average=52,748) applying purified IgG in the 72-plexed Luminex-bead-array.

Correlation of Luminex and macro-array data

Comparison of reactivities on macro-array and Luminex platform shows the highest correlation coefficient (r=0.56) with purified IgG samples and native Luminex assay conditions. Under denaturating conditions, the Luminex data give no correlation with the microarray data, independent for both sample types (purified IgG or serum dilution). Although comparison of native and denaturating assay conditions of the Luminex bead-array measurements show high correlation coefficients of r=0.75 and r=0.90 measured with purified IgG and 1:200 serum dilution, respectively. Native conditions seem advantageous for the analysed antigens (Figure 4).

Class Comparison Analysis

Class Comparison analysis of the seronegative (n=16) and sero-positive (n=16) RA sample set tested in the 72-plexed Luminex-bead-array revealed a potential marker for the seronegative RA samples which shows higher MFI signals in comparison to sero-positive RA (Fold-change=1.95 at p<0.05).

Class Comparison Analysis of the RA-positive IgG samples provided by *engine* (n=24) versus healthy controls (n=15) from the independent sample set resulted in 26 significant markers (p<0.05), 16 tested under "denatured" and 10 tested under "native" conditions (Figure 5).

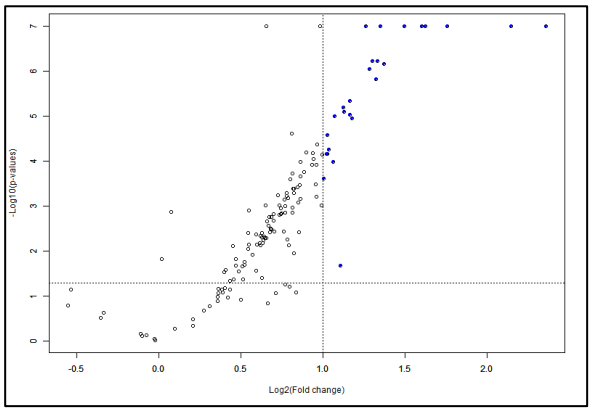

Figure 5: Volcano plot derived from correlation analysis of 8 RA positive (provided by engine) and 15 healthy control samples. 26 potential markers show significant differential reactivity between RA-positive and healthy control samples at p<0.05.

Conclusion

The Luminex bead-based assay enables confirmation and validation of multiple candidate protein/markers (up to 500) from the same sample simultaneously and thereby requires only a small amount of sample (<5 µL serum/plasma or 5 µg purified IgG). Analytical sensitivity, linear dynamic measurement ranges and assay reproducibility are outperforming microarray analysis.

In this study normalized amounts of purified IgG tested with native Luminex assay condition shows the highest concordance between macro-arrays and Luminex bead-array data. Although higher correlation between native and denatured Luminex assay conditions were obtained when using diluted serum, this increased correlation compared to values from purified IgG might

be caused by potential background or matrix effects from serum. Therefore, purified and concentration standardised IgG gives more reliable results as shown in our previous studies [4]. The Luminex bead-array analysis can be conducted in 96 and 384 well formats, thus paralleled analysis of several hundred samples per day can be conducted easily. This multiplexed bead-array analysis is currently the most efficient approach for selecting the best antigenic markers during confirmation studies using >250-plexed assays and high sample numbers of >50-200 samples per clinical (sub)-group to provide sufficient statistical power.

The combination of macro-arrays based screening and subsequent selection and confirmation of antigenic proteins on Luminex-bead-arrays is a highly efficient strategy for biomarker research and development.

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