

## MicroBioChips Service Report

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Project # TRYMBCINV0009

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**I - Summary**

Four antibodies produced in xyz plus a negative control were incubated on Invitrogen's Human ProtoArray v4.1. The purpose of the experiment was to select the proteins presenting the highest specific reactivity with each antibody. All samples demonstrated good signal to background ratio on the arrays and the controls were correct. The comparison of the antibodies' reactivity, achieved from the Z-score values and Hit classification, allowed to select a number of reactive proteins to each tested antibody.

The table below displays the proteins found to show a high specific reactivity with **Ab1** Antibody selected with the different calculation methods (Global & MBC)

MBC calculation method results:

Name	Ab1 MBC processed net value	Protein Header
Prot1 Ref.	7.0	Prot1 name
Prot2 Ref.	4.0	Prot2 name
Prot3 Ref.	3.7	Prot3 name
Prot4 Ref.	2.7	Prot4 name
Prot5 Ref.	2.4	Prot5 name
Prot6 Ref.	1.8	Prot6 name
Prot7 Ref.	1.5	Prot7 name

Global calculation method results:

Name	Ab1 Global processed net value	Protein Header
Prot1 Ref.	4.9	Prot1 name

The table below displays the proteins found to show a high specific reactivity with **Ab2** Antibody selected with the different calculation methods (Global & MBC)

MBC calculation method results:

Name	Ab1 MBC processed net value	Protein Header
ProtA Ref.	6.0	ProtA name
ProtB Ref.	4.5	ProtB name
ProtC Ref.	4.1	ProtC name
ProtD Ref.	3.8	ProtD name
ProtE Ref.	2.3	ProtE name
ProtF Ref.	2.2	ProtF name
ProtG Ref.	1.9	ProtG name

Global calculation method results:

Name	Ab1 Global processed net value	Protein Header
ProtB Ref.	3.2	ProtB name

The table below displays the proteins found to show a high specific reactivity with **Ab3** Antibody selected with the different calculation methods (Global & MBC)

MBC calculation method results:

... Table displaying the results ...

Global calculation method results:

... Table displaying the results ...

The table below displays the proteins found to show a high specific reactivity with **Ab4** Antibody selected with the different calculation methods (Global & MBC)

MBC calculation method results:

... Table displaying the results

Global calculation method results:

... Table displaying the results ...

## II - Introduction

Functional protein microarrays (ProtoArrays™) are new tools that empower investigators with defined high-protein content for rapidly screen antibodies of interest for interaction with thousands of proteins. Invitrogen's ProtoArray™ Human Protein Microarrays contain as many as 8,000 purified human proteins immobilized on nitrocellulose-coated glass slides. The ProtoArray human protein microarrays contain proteins that are expressed in insect cells, and therefore are expected to contain appropriate posttranslational modifications. Since all proteins are purified under native conditions, immobilized proteins are expected to maintain their native conformations. Compared to other methods, the protein microarray offers several advantages, including rapid screening and high sensitivity. Since the identity of every protein printed on the protein array is known, any antibody cross-reactivity with proteins identified on the array can be queried to identify common sequence motifs that might explain cross-reactivity.

An overview of the Antibody Profiling Service provided by MicroBioChips is shown in figure 1.

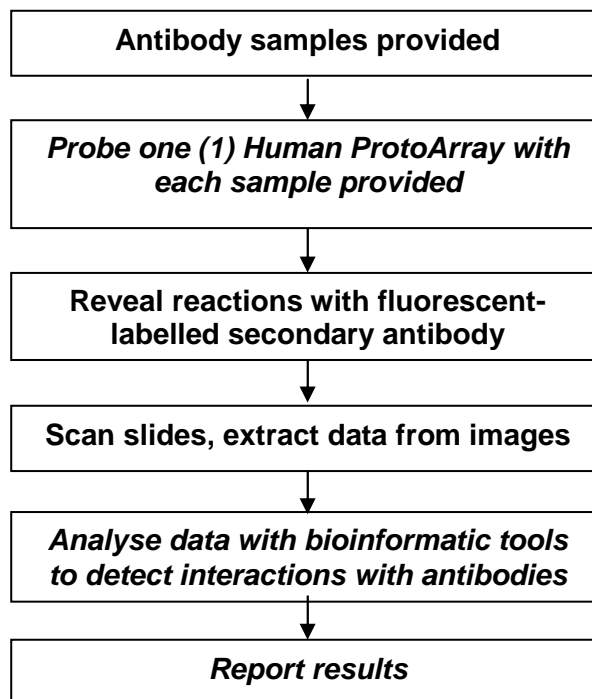


Fig. 1: overview of the Antibody Profiling Service.

## III – Material and method

### III – 1 Experimental Design

In this study 4 antibodies samples and one control (for AlexaFluor647-anti-mouse secondary antibody reactivity assessment) were profiled on ProtoArray™ Human Protein Microarrays v4.1 containing more than 8,000 human proteins. Antibodies were profiled at a **xxµg/mL** dilution utilizing one ProtoArray™ per sample.

### III – 2 Assay Controls

The purpose of these control proteins is to provide reference points for data acquisition and analysis. The AlexaFluor conjugated antibody allows proper alignment of the spot finding software for data acquisition. Each ProtoArray™ subarray contains a gradient of human IgG (boxed in white, Fig. 2 below), which serves as a control for proper performance of the detection reagent (IRBP application, use of AlexaFluor® anti-Human Ab). Each ProtoArray™ contains two proteins designed to serve as positive controls for the performance of the Immune Response Biomarker Profiling assay (IRBP). Anti-human IgG antibody is spotted in two locations on the array and binds to IgG present in the serum sample. Additionally, ProtoArrays™ contain influenza A spotted in two locations (not displayed here). Invitrogen has demonstrated that ≥95% of serum samples are immunoreactive against the influenza A antigen.

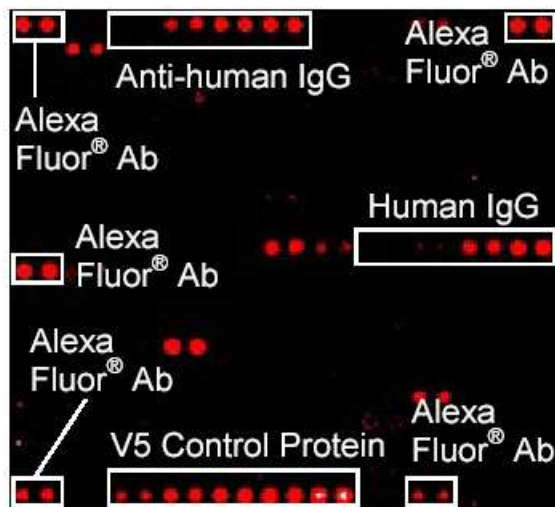


Fig. 2: Image from an assay control results – control spots on the Human ProtoArray (IRBP application).

### III – 3 Blocking and Probing protocol for ProtoArray v4.0

Microarray slides (same **Batch #00000**) were blocked in x ml Blocking Buffer (...- Buffer composition description - ...) in array chambers for x hour at xx°C with gentle shaking (25 rpm). After blocking the array was allowed to incubate for xx minutes at xx°C with x mL of each antibody sample diluted at xxµg/mL in the probing buffer (- Buffer composition description -), ...

... All the incubation steps for the antibody samples and control are described in detailed here...

### III – 4 Data Acquisition

The slides were scanned with an InnoScan 700 scanner

The following scanning parameters were set on the Mapix software for each array scanned:

Laser Power : **635 - xxx** / 532 – xxx  
PMT : **635 - xxx** / 532 – xxx  
Resolution : **xx**  $\mu$ m / pixel

Image analysis parameters sets for each array :

	Valeur
Diamètre fixe =Dth	NON
Proportion de spots visibles en %	90
Erreur position Max (%Pas)	30
Diamètre Min (%Dth)	80
Diamètre Max (%Dth)	150
Largeur frontière S/B (pixels)	2
Diamètre Background (pas)	2

Mapix 2.8.2 software was used to overlay the mapping of human proteins in the array list file to each array image. After aligning each of the 48 subarrays using spots from the AlexaFluor-conjugated and murine antibodies printed in each subarray, pixel intensities for each spot on the array were determined from the software and saved to a text and gpr (genepix) file. These files can be opened in Cluster, Treeview, Excel, Prospector, and other text editing or spreadsheet programs.

Quantitated spot files were processed to determine which proteins interact with the antibody samples.

### IV – Controls analysis

***All the raw and treated data are available on the CD/DVD delivered with this report.***

**IV – 1 Quality control on each Array**

Quality controls are present in each 48 blocks of the protoarray. The table below displays the average reactivity values for all the controls present on the array. This table is available on the file “controls.xls” (see the delivered CD-rom).

Population	Protein Amount	Mean Control	Mean Ab1	Mean Ab2	Mean Ab3	Mean Ab4
AlexaAntiMouseAb	0	14166.3	22641.2	26313.3	25982.2	20157.1
Anti-HumanIgA1	0	0.7	0.9	1.9	3	2.7
Anti-HumanIgA2	0	2.2	4.8	3.3	4.1	7.4
Anti-HumanIgG1	0	4.1	62.9	45.6	50	58.4
Anti-HumanIgG2	0	5.6	152.9	139.8	161.8	202.7
Anti-HumanIgG3	0	8.1	520.6	534.8	634.7	732.7
Anti-HumanIgG4	0	55.3	2275.3	2443.9	2866.4	3174.4
BSA	0	8.9	8.9	5.5	7.2	11.8
Buffer	0	9.3	8.1	7	7	10
ExtraBuffer	0	18.9	31.8	32.5	32.7	32.5
GST1	5036.53	17.7	13.5	10.7	9.1	16
GST2	12225.4	2.4	3.7	3.5	4.1	1.9
GST3	32292.1	7.1	5.6	6.9	4.3	6.1
GST4	64752.1	6.9	4.4	10	3.6	5.7
HumanIgA1	0	48.9	45.4	40.6	44.2	44.2
HumanIgA2	0	178.5	136.6	144.5	146.3	145.3
HumanIgG1	0	20.3	21.5	20.8	23.9	24.2
HumanIgG2	0	46.5	41.4	38.9	33.3	39.6
HumanIgG3	0	168	132.1	134.6	144.5	137.5
HumanIgG4	0	680.7	569.4	559.4	586.7	546
V5control1	0	8.9	18.4	16.2	16	14.6
V5control2	0	3.3	7	7	7.4	4.9
V5control3	0	4.4	6.5	7.4	5.1	7.8
V5control4	0	6.3	3.6	6	3.9	3.7
V5control5	0	4.3	3.6	3.9	3.8	3.6
All Negative Controls	N/A	10.7	12.1	11.7	11.1	13.5
Probes	N/A	70.4	79.5	62.4	68.6	67.2

Fig. 5 : Table displaying the average reactivity measured for the controls for each incubated sample.

The AlexaAntiMouse Ab showed as expected high signals for all samples. The Anti-HumanIgG spots showed a slight reactivity with the antibodies from xyz, particularly for the most concentrated spot (anti-humanIgG4), This only shows that these spotted antibodies cross-react with xyz IgG. The spotted Human IgG&A did not showed any relevant reactivity. The negative controls (BSA, Buffer, GST) showed as expected low reactivity.

**All the Arrays experiments were validated for further analysis.**

**IV – 2 ProtoArray external protein controls.**

Other external controls some of them known as auto-antigens are present on the arrays. The following table (Fig. 6 next page) and graphic (Fig. 7 next page) displays the reactivity (duplicate mean net signal = signal of the spot-Background around the spot) of these controls with the tested antibodies and control. These results are also available in the file “External-controls.xls”.

Name	Duplicate mean net signal				
	Ab1	Ab2	Ab3	Ab4	Control
CTL2127	9.5	16.25	29.25	19	18.25
CCP_10BSA	-16	-0.5	5.5	-10.75	5.5
CCP_1BSA	-4.5	-5	22	-1	4.25
myeloperoxidase_Sigma	12.25	-7.25	-8.25	38.75	-2.25
dsDNA_plasmid	11.5	0.5	-5.25	-2	0
VIMENTIN	1	28	22.5	7.5	-5.25
U1snRNP68	7.5	10.5	8.5	16.5	-15
La	6	14	-3.5	3	-8
RNP_COMPLEX	7.5	25	13	26.5	16.25
histone	136.75	183.75	109	87	43.5
Topol	1335	1202.5	1138	1292	1317.5
Histone_F2a2	306.25	297.5	827.75	193	172.5
ssDNA	-19.25	16.25	33	-2.5	1.25
CENPB	12.5	57.25	6	11	7
TRANSGLUTAMINASE	4	-2.5	2.75	-2	1
rRNA	9.5	65.5	59.25	2	0.5
JO-1	30	-2	-4	-4.5	17.5
dsDNA_genomic	4.5	-2	-5	-7	-1.5
CARDIOLIPIN	5.25	1.5	4	2.25	-8.25
thyroglobulin	3.75	-15	8	0	22.5
Ro-52	78.75	41	18.5	30.5	21.25
RNA_POLYMERASE	8.25	16.5	3.25	23	10.5
PYRUVATE_DEHYDROGENASE	-0.5	1.75	-6	5.75	1.5
proteinase-3_Sigma	1.25	6	-1	6.5	2
myeloperoxidase_IV	-1.75	-13	-8	-1	-21.5
influenza_A	127.5	30.5	50.5	84	63.25

Fig. 6 : Net signal (duplicate mean of median signal of each spot - background signal around each spot) of each sample with the externals controls on the Array (26 controls).

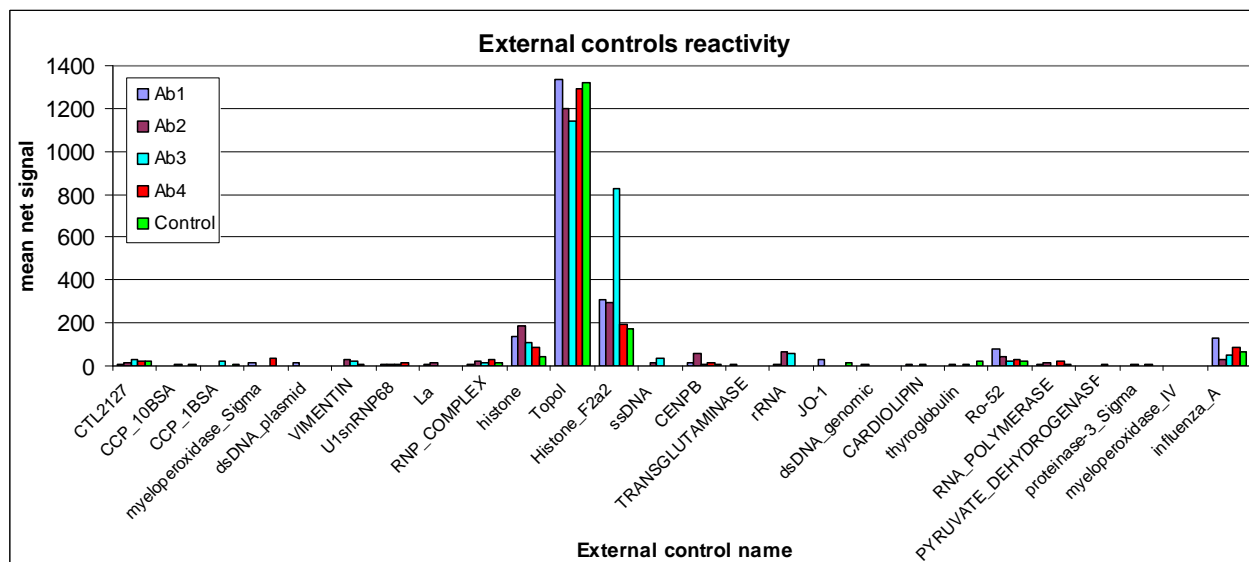


Fig. 7 : Graphic displaying the reactivity of the external controls with the incubated antibodies and controls.

**V – “protein amount” parameter on invitrogen’s protoarray slides**

... - this part explains what is the protein amount parameter displayed for most spotted protein on protoArrays slides - ...

**VI – Data treatment**

There are substantial differences between ProtoArrays™ and other array-based technologies currently employed for biomarker discovery, including DNA microarrays. It is useful to note the difference in data types that result from each of these approaches. DNA microarrays generate a range of values in which the signal intensity is thought to correspond directly to the number of transcripts. Protein microarrays generate data that must be evaluated for the presence or absence of a significant signal. These two data types, known as continuous numerical data and dichotomous indicator data respectively, necessitate fundamentally different statistical approaches.

... Description of the Data treatment method ... and description of the different steps :

1. Process data : ...Description...
2. Assign “Hits” : ...Description of Hits...
3. Assign indexes : ...Description of Index...
4. Identify differentially immunoreactive proteins: ...Method to differentiate reactivity...

**VI – 1 Influence of the spotted protein amount on signal distribution.**

...Description of the influence of protein amount on signal, graphical description...

...Description of the influence of protein amount on signal, and graphical description...

## **VI – 2 Microbiochips' (MBC) data processing method**

... Description of MBC data processing method, with graphical demonstration ...

## **VI – 3 Global data processing method**

... Description of Global data processing method...

... Description of the colour code (flagged/coloured data)

**VI – 4 Comparison of the reactivity and selection of the proteins presenting a specific reactivity**

... Description of the protein sorting method... and coloured flag code...

Some proteins react directly with the secondary antibody (control). These protein were removed from the final tables. This led to the following tables for each tested antibody (Fig. 10).

Fig. 10 = Table displaying the reactivity of the protoArrays' protein for Ab1 antibody (which is an extract of the complete tables available on the delivered CD/DVD), containing the processed values, "Hits" value, comparison of Ab1 reactivity with control and other Antibody tested

**VII – comparison results**

**VII –1 Proteins selection criteria**

The proteins were sorted and selected upon their processed values...

... Description of the protein selection method ...

...The list of selected proteins in global and MBC calculation method were compared, the proteins selected by both methods were underlined in **light blue**.

**VII - 2 Specific reactivity of Ab1 antibody**

The table below (Fig. 13) shows the proteins selected with the global calculation, and the next Fig. 13b displays the detailed name and references of the selected proteins.

Name	Ab1-global-processed duplicate mean	Ab2-global-processed duplicate mean	Ab3-global-processed duplicate mean	Ab4-global-processed duplicate mean	Control-global-processed duplicate mean	Ab1 Hits	Ab2 Hits	Ab3 Hits	Ab4 Hits	Control Hits	Ab1 Global processed net value
<u>Prot1 Reference</u>	4.9	0.0	0.1	0.0	0.0	1	0	0	0	0	4.9

Fig. 13 : This is the protein showing the best specific reactivity with Ab1 antibody. Protein selected upon the selection criteria, the protein underlined in blue is also selected with the MBC calculation process (see file "Ab1-Global-Process-selection.xls" sheet "Ab1-Selection").

Acc N°	ORF id	SwissProt id	Gene Symbol	Protein Header
<u>Prot1 Ref.</u>	xxx	xxx	xxx	Prot1 name

Fig. 13 b : Detailed proteins ID displayed in the previous table.

The tables below (Fig. 14 & 14b) display the proteins selected with the **MBC data processing**.

Name	Ab1-MBC-processed duplicate mean	Ab2-MBC-processed duplicate mean	Ab3-MBC-processed duplicate mean	Ab4-MBC-processed duplicate mean	Control-MBC-processed duplicate mean	Ab1 Hits	Ab2 Hits	Ab3 Hits	Ab4 Hits	Control Hits	Ab1 MBC processed net value
<u>Prot1 Ref.</u>	6.8	-0.1	-0.1	-0.2	-0.2	1	0	0	0	0	7.0
Prot2 Ref.	3.8	3.8	1.8	1.4	-0.2	1	1	0	0	0	4.0
Prot3 Ref.	3.7	4.4	3.6	5.0	0.0	1	1	1	1	0	3.7
Prot4 Ref.	3.1	0.0	1.1	0.8	0.5	1	0	0	0	0	2.7
Prot5 Ref.	4.0	4.1	2.4	4.2	1.6	1	1	0	1	0	2.4
Prot6 Ref.	1.5	0.6	0.9	0.9	-0.3	0	0	0	0	0	1.8
Prot7 Ref.	1.3	-0.3	0.4	1.0	-0.2	0	0	0	0	0	1.5

Fig. 14: These are the proteins showing the best specific reactivity with Ab1 antibody (MBC calculation process). Protein selected upon the selection criteria, the protein underlined in blue was also selected with the global calculation process (see file “Ab1-MBC-Z-score-selection.xls” sheet “Ab1-Selection”).

Acc N°	ORF id	SwissProt id	Gene Symbol	Protein Header
<u>Prot1 Ref.</u>	xxx	xxx	xxx	Prot1 name
Prot2 Ref.	xxx	xxx	xxx	Prot2 name
Prot3 Ref.	xxx	xxx	xxx	Prot3 name
Prot4 Ref.	xxx	xxx	xxx	Prot4 name
Prot5 Ref.	xxx	xxx	xxx	Prot5 name
Prot6 Ref.	xxx	xxx	xxx	Prot6 name
Prot7 Ref.	xxx	xxx	xxx	Prot7 name

Fig. 14 b : Detailed proteins ID displayed in the previous table.

.... Comments about these results ....

The detailed ID of the proteins are available in the file “V4.1\_proteinInfo.xls” in the delivered CD.

**VII - 3 Specific reactivity of Ab2 antibody**

....Tables of results like for Ab1 and comments about the selected proteins...

#### **VII - 4 Specific reactivity of Ab3 antibody**

...Tables of results like for Ab1 and comments about the selected proteins...

#### **VII - 5 Specific reactivity of Ab4 antibody**

...Tables of results like for Ab1 and comments about the selected proteins

#### **VIII – Conclusion**

... Discussion about the results...

This protein array analysis, based upon two processing methods and hits calculation process, with the defined selection criteria, allowed to identify the most reactive target antigens.

The MBC calculation process allowed to select more proteins and define a more effective antibody profiling. The proteins selected on the basis of global calculation were also found in the MBC processing method.