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1 ABOUT SENGENICS

Sengenics is a Functional Proteomics company that was originally spun out from research carried out at Cambridge University in the UK. The company has a patented technology called KREX™ which has world renowned customers and collaborators that include top pharma, biotech and ivy league academic institutions in the USA, Europe and Asia.

2 KREX™ TECHNOLOGY

KREX™ is the world’s only proteomics technology that produces full-length, correctly folded, functionally verified proteins. KREX™ utilises Biotin Carboxyl Carrier Protein (BCCP) folding marker which is cloned in-frame with the gene encoding the protein of interest. BCCP acts not only as a protein folding marker but also as a protein solubility enhancer.

BCCP is fused to either the N- or C-terminal of a protein of interest. Full-length proteins are expressed as fusions to the BCCP folding marker which becomes biotinylated in vivo, BUT only when the protein is correctly folded. Conversely, misfolded proteins drive the co-translational misfolding of BCCP to such an extent that it becomes catalytically inactive, and is unable to become biotinylated. Hence, misfolded proteins no longer have a way of attaching to a streptavidin-coated solid support. This is the fundamental principle of the KREX™ technology. Only correctly folded proteins become attached to a solid support.

The Fundamental Principle of KREX™

01 Protein folds correctly
02 BCCP Biotinylated
03 Protein immobilised
04 Misfolded protein
05 BCCP not biotinylated
06 Protein cannot stick
As biotinylated proteins bound to a streptavidin-coated surface show negligible dissociation, this interaction therefore provides a vastly superior means for tethering proteins to a planar surface and is ideal for applications such as protein arrays, SPR and bead-based assays. The use a compact, folded, biotinylated, 80 residue domain BCCP affords two significant advantages over the AviTag and intein-based tag. First, the BCCP domain is cross-recognised by eukaryotic biotin ligases enabling it to be biotinylated efficiently in yeast, insect, and mammalian cells without the need to co-express the E. coli biotin ligase. Second, the N- and C-termini of BCCP are physically separated from the site of biotinylation by 50Å, so the BCCP domain can be thought of as a stalk which presents the recombinant proteins away from the solid support’s surface, thus minimising any deleterious effects due to immobilisation.

The success rate for KREX™ mediated expression of even the most complex proteins is in excess of 98%. The technology can be applied in a highly parallelised pipeline resulting in high-throughput, highly consistent production of functionally validated proteins. This ensures that only the correct final 3-dimensional structure is presented on solid supports for truly meaningful biological interactions.

One critical factor that determines the quality of results is the surface chemistry of the solid support the protein is immobilised onto. An ideal surface chemistry should resist non-
specific adsorption, provide sufficient stability for the three-dimensional structure of the protein and also provide an environment that allows the protein to retain its native conformation and functionality. As proteins have numerous hydrophobic domains and charged sites, they tend to adsorb non-specifically to most solid surfaces which often results in the disruption of their 3-D structure and eventually, complete loss of activity. This is circumventable using KREX™. Proteins produced using KREX™ technology can be immobilised onto a wide variety of streptavidin-coated PEG-derivatised solid supports such that they retain folded structure and function in an aqueous environment, and behave in miniaturised, highly multiplexed quantitative assays as if they are in free solution.

Addition of BCCP permits the monitoring of fusion protein folding by measuring the extent of in vivo biotinylation. This can be measured by standard blotting procedures, using SDS-PAGE or in situ colony lysis and transfer of samples to a membrane, followed by detection of biotinylated proteins using a streptavidin conjugate such as streptavidin-horseradish peroxidase.

Additionally, the fact that the BCCP domain is biotinylated in vivo is particularly useful when attempting to multiplex protein purification for fabrication of protein arrays since the proteins can be simultaneously purified from cellular lysates and immobilised in a single step via the high affinity and specificity exhibited by a streptavidin surface.

### TECHNICAL PERFORMANCE

#### Sensitivity

**Definition:**

FDA definition of “sensitivity” is an estimate of the lowest concentration of an analyte that can be measured, i.e., the limit or threshold of detection on the array above a defined cut-off (in this case, two standard deviations of the background).
Purpose:
To determine the optimal serum and anti-human IgG antibody dilutions for the detection of antigen binding on the array.

Observations:
A linearity assay of a given serum sample at a 20µg/mL concentration secondary antibody revealed a linear response over greater than three orders of magnitude (Figure 5a and 5c). The limit of detection of approximately 1:1,000,000 serum dilution was identified. A second linearity assay observed a detection limit of an autoantibody titer of 190 pg/mL on the array (Figure 5b).

Figure 5. Linearity and dynamic range assays of autoantibody detection on the array surface. (a) Initial linearity assay using a series of serum dilutions of patient samples, using 20 µg/mL of secondary Ab for detection. Inset: Scatchard plot of the same data. (b) A second linearity assay using two serum dilutions (1:200 and 1:2,400) of the same patient sample with a range of secondary Ab concentrations to determine. (c) A dynamic range assay indicating the linear range of the platform. The limit of detection (approximately 1:1,000,000 serum dilution) is where the data intersects with the signal cut-off level (two standard deviations of the background; indicated here as the dashed line). [Beeton-Kempen N. et al., 2014]
Specificity

Definition:
The ability of a given probe to bind to a particular substance of interest rather than other materials in a given sample.

Purpose:
To investigate the binding selectivity of each protein immobilised onto the array surface for content verification and functionality.

i) Experiment A:
Two specificity assays were run on our p53 Protein Function Array;
   1. Cy5-anti-p53 Antibody Assay
   2. Cy3-GADD45 DNA Assay

The p53 Protein Function Array contains 1 wild-type human p53 protein, 45 mutant p53 proteins and 8 Cy3-labelled BSA marker printed in duplicate. A scanned image of the slide ran on Assay 1 above shows a visualisation only p53 probes (Figure 6a). A scanned image of the slide ran on Assay 2 above can be seen in Figure 6b, showing an expected pattern of GADD45-p53 DNA binding.
ii) Experiment B:

At the presence of ATP, some protein kinases will autophosphorylate. Array-based autophosphorylation assays were run on the Protein Kinase Array (contains 80 human protein kinases). Figures 7a and 7b show scanned images of our Protein Kinase Arrays with and without the presence of ATP respectively, showing autophosphorylation activities on known protein kinases.
Figure 7. Array-based autophosphorylation assays. Here, an array of 80 human protein kinases was printed in duplicate and assayed for autophosphorylation activity. The assays revealed an expected pattern of ATP-dependent, on-array autophosphorylation, as marked. (a) Kinase buffer only. (b) Kinase buffer containing 100µM ATP. [Blackburn JM & Shoko A 2011]
Accuracy

Definition:
FDA standard defines “accuracy” as a measure of how close a measured value is to the true value. A true value may be defined as either;

1. Reference materials with known concentrations or activities.
2. Well-recognised reference methods which have proven to deliver valid results.

Purpose:
To investigate and validate the accuracy of identified antigens using the protein microarray by comparison with a standard serological standard method, ELISA.

Observations:
Confirmation of correlation between protein array results and ELISA from the comparison analysis of non-small cell lung cancer (NSCLC) patient serum versus healthy serum; 7 antigens were tested with 53 samples and observed a correlation in 94% of the cases (Figure 8).

Recommendations:
In the case of our protein array, one of the challenges faced in determining the accuracy of our assay and platform based on Definition 1 of a “true value” above is the high variability of autoantibody titers between samples in either serum or plasma towards each protein on the array. For this reason, an optimisation of sample dilution will be performed prior to each study to reduce the occurrence of any non-specific binding or matrix effects, thus improving the accuracy of the results.
Figure 8. A total of 53 healthy donor sera (top) and NSCLC sera (bottom) were tested against 7 antigens by ELISA (results expressed in reciprocal titers) or by Seromics (results expressed as the ratio of observed reactivity to the cut-off). Bold characters indicate concordance. [Gnjatic S et al., 2009]
**Precision**

**Definition:**
FDA standard defines “precision” as a measurement of variability between repeated measurements.

**Purpose:**
To identify the consistency of proteins and control probes between and within protein array slides by measure of CV%.

**Observations:**
After data pre-processing, the CV% of each replica spot or protein within slides should not exceed 20%. Proteins or spots with CV% > 20% will be “flagged”.
The average CV% of all replica spots or proteins within slides should not exceed 10%.
The number of proteins or probes within a slide with CV% > 20% should not exceed 5%.

<table>
<thead>
<tr>
<th>Pooled Negative Sample Run</th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Expt 3</th>
<th>Expt 4</th>
<th>Expt 5</th>
<th>Expt 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average CV%</td>
<td>5.339%</td>
<td>6.173%</td>
<td>5.547%</td>
<td>6.008%</td>
<td>4.982%</td>
<td>5.390%</td>
</tr>
</tbody>
</table>

Table 1. The average CV% of all proteins and control probes within each sample for 6 repeated experiments using the same sample (Pooled Negative Sample). [Sengenics Data]
Figure 9. The CV% for all proteins and control probes within slide for Experiment 1. [Sengenics Data]

Figure 10. The CV% for all proteins and control probes within slide for Experiment 2. [Sengenics Data]
Figure 11. The CV% for all proteins and control probes within slide for Experiment 3. [Sengenics Data]

Figure 12. The CV% for all proteins and control probes within slide for Experiment 4. [Sengenics Data]
Figure 13. The CV% for all proteins and control probes within slide for Experiment 5. [Sengenics Data]

Figure 14. The CV% for all proteins and control probes within slide for Experiment 6. [Sengenics Data]
Figure 15. The CV% for all proteins and control probes between 6 repeated experiments using the same sample (Pooled Negative Sample). [Sengenics Data]
KREX™ PROTEIN ARRAYS & PROTEIN LIBRARY

IMMUNOME™ v4 Discovery Array is a protein array which utilises the patented Sengenics KREX™ functional proteomics technology. All proteins on the IMMUNOME™ v4 Discovery Array are correctly folded and functional as they are expressed as fusion proteins with biotin carboxyl carrier protein (BCCP) which acts as a folding marker and solubility enhancer. The IMMUNOME™ v4 Discovery Array contains 1600+ full-length human proteins from biologically significant protein families including kinases, signalling molecules, cytokines, interleukins, chemokines and cancer antigens. Each protein on the array is spotted in quadruplicate. The arrays are coated with a proprietary surface which preserves protein folding and inhibits binding of non-specific proteins.

Applications:

- Discovery of autoantibody biomarkers for diagnostics
- Discovery of autoantibody biomarkers of risk of adverse drug reactions
- Discovery of autoantibody-based therapeutic and prophylactic biologics
- Determining effect of citrullination on autoantibody interactions
- Functional assays for any protein or any interacting biomolecule

Protein List:

A full list of the proteins available on the IMMUNOME™ v4 Discovery Array can be found here: https://www.sengenics.com/protein-list/

No. of Sample run per Slide

1

Control included

- Cy3BSA-1 – 23
- IgG-1 – 6
- IgM-1 – 6
- IgA-1 – 6
- ZZ_CON1
- ZZ_CON2
- ZZ_CON3
- ZZ_CON4
CTA Array

CTA Array is a protein array which utilises the patented Sengenics KREX™ functional proteomics technology KREX™-based protein array for high-throughput quantification of autoantibodies to Cancer-Testis (CT) antigens. All proteins on the CTA Array are correctly folded and functional as they are expressed as fusion proteins with biotin carboxyl carrier protein (BCCP) which acts as a folding marker and solubility enhancer. The CTA Array contains over 200 full-length CT antigens and cancer-related antigens that show highly restricted expression profiles in adult somatic tissues but which are aberrantly expressed in various cancers. Each protein on the array is spotted in triplicate. The arrays are coated with a proprietary surface which preserves protein folding and inhibits binding of non-specific proteins.

Applications:

- Unique differential profiling of cancer-antigen in various cancer types
- Patient stratification in checkpoint inhibitor studies
- Determination of epitope recognition in patients or monitor patients in experimental cancer vaccine studies

Protein List:

A full list of the proteins available on the CTA Array can be found here: [https://www.sengenics.com/protein-list/](https://www.sengenics.com/protein-list/)
No. of Samples run per Slide:

4

Controls included:

- Cy3BSA-1 – 12
- IgG-1 – 6
- IgM-1 – 6
- IgA-1 – 6
- ZZ_CON1
- ZZ_CON2
- ZZ_CON3
- ZZ_CON4

Layout:
OncoREX p53 Cancer Array

OncoREX p53 Cancer Array is a protein array which utilises the patented Sengenics KREX™ functional proteomics technology KREX™-based protein array for high-throughput quantification of autoantibodies to wild-type and mutant p53. All proteins on the oncoREX p53 Cancer Array are correctly folded and functional as they are expressed as fusion proteins with biotin carboxyl carrier protein (BCCP) which acts as a folding marker and solubility enhancer. The oncoREX p53 Cancer Array contains a total of 1 wild-type and 99 p53 mutants. Each protein on the array is spotted in triplicate. The arrays are coated with a proprietary surface which preserves protein folding and inhibits binding of non-specific proteins.

Applications:

- Characterise the effect of 100 different p53 isoforms on its DNA binding activity
- Discovering new compounds that can restore the function of mutant p53
- Determine the effect of p53 mutations on binding of autoantibodies and antibodies

Protein List:

A full list of the proteins and protein variants available on the oncoREX p53 Cancer Array can be found here: https://www.sengenics.com/protein-list/
No. of Samples run per Slide:

4

Controls included:

- Cy3BSA-1 – 12
- IgG-1 – 6
- IgM-1 – 6
- IgA-1 – 6
- ZZ_CON1
- ZZ_CON2
- ZZ_CON3
- ZZ_CON4

Layout:
Sengenics KREX List of Controls

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy3BSA-1</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 1; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
</tr>
<tr>
<td>Cy3BSA-2</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 2; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 3; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<td>Cy3BSA-4</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 4; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<tr>
<td>Cy3BSA-5</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 5; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<td>Cy3BSA-6</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 6; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<td>Cy3BSA-7</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 7; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<td>Cy3BSA-8</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 8; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<td>Cy3BSA-9</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 9; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<td>Cy3BSA-10</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 10; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<td>Cy3BSA-11</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 11; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<td>Cy3BSA-12</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 12; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<td>Cy3BSA-13</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 13; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<td>Cy3BSA-14</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 14; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 15; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment;</td>
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<tr>
<td>Cy3BSA</td>
<td>Description</td>
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<td>Cy3BSA-16</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 16; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<td>Cy3BSA-17</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 17; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<td>Cy3BSA-18</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 18; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<td>Cy3BSA-19</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 19; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<td>Cy3BSA-20</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 20; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<tr>
<td>Cy3BSA-21</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 21; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
</tr>
<tr>
<td>Cy3BSA-22</td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 22; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
</tr>
<tr>
<td><strong>Cy3BSA-23</strong></td>
<td>Cy3-labelled Biotinylated Bovine Serum Albumin replica 23; Acts as a positive control on each array of the slide; Binds to the fluorescent dye used in the experiment; Their concentrations are kept constant throughout all slides and all replicas; Also used for downstream normalisation of signal intensities for any given study using the array.</td>
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<tr>
<td><strong>IgG-1</strong></td>
<td>Immunoglobulin G Dilution 1; Used to assess the binding capacity of fluorescent-conjugated secondary incubation; Acts as a positive control when Cy3-anti human IgG secondary antibody is used in the experiment; Acts as a negative control when other secondary antibodies (e.g. anti-human IgA, anti-human IgM etc.) are used in the experiment;</td>
</tr>
<tr>
<td><strong>IgG-2</strong></td>
<td>Immunoglobulin G Dilution 2; Its concentration on the arrays is half of the concentration of control IgG-1; Used to assess the binding capacity of fluorescent-conjugated secondary incubation; Acts as a positive control when Cy3-anti human IgG secondary antibody is used in the experiment; Acts as a negative control when other secondary antibodies (e.g. anti-human IgA, anti-human IgM etc.) are used in the experiment;</td>
</tr>
<tr>
<td><strong>IgG-3</strong></td>
<td>Immunoglobulin G Dilution 3; Its concentration on the arrays is half of the concentration of control IgG-2; Used to assess the binding capacity of fluorescent-conjugated secondary incubation; Acts as a positive control when Cy3-anti human IgG secondary antibody is used in the experiment; Acts as a negative control when other secondary antibodies (e.g. anti-human IgA, anti-human IgM etc.) are used in the experiment;</td>
</tr>
<tr>
<td><strong>IgG-4</strong></td>
<td>Immunoglobulin G Dilution 4; Its concentration on the arrays is half of the concentration of control IgG-3; Used to assess the binding capacity of fluorescent-conjugated secondary incubation; Acts as a positive control when Cy3-anti human IgG secondary antibody is used in the experiment; Acts as a negative control when other secondary antibodies (e.g. anti-human IgA, anti-human IgM etc.) are used in the experiment;</td>
</tr>
<tr>
<td><strong>IgG-5</strong></td>
<td>Immunoglobulin G Dilution 5; Its concentration on the arrays is half of the concentration of control IgG-4; Used to assess the binding capacity of fluorescent-conjugated secondary incubation; Acts as a positive control when Cy3-anti human IgG secondary antibody is used in the experiment; Acts as a negative control when other secondary antibodies (e.g. anti-human IgA, anti-human IgM etc.) are used in the experiment;</td>
</tr>
<tr>
<td><strong>IgM-6</strong></td>
<td>Immunoglobulin G Dilution 6;</td>
</tr>
<tr>
<td>Dilution</td>
<td>Description</td>
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<tr>
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</table>
| IgM-1     | Immunoglobulin M Dilution 1;  
Used to assess the binding capacity of fluorescent-conjugated secondary incubation;  
Acts as a positive control when Cy3-anti human IgM secondary antibody is used in the experiment;  
Acts as a negative control when other secondary antibodies (e.g. anti-human IgG, anti-human IgA etc.) are used in the experiment; |
| IgM-2     | Immunoglobulin M Dilution 2;  
Its concentration on the arrays is half of the concentration of control IgM-1;  
Used to assess the binding capacity of fluorescent-conjugated secondary incubation;  
Acts as a positive control when Cy3-anti human IgM secondary antibody is used in the experiment;  
Acts as a negative control when other secondary antibodies (e.g. anti-human IgG, anti-human IgA etc.) are used in the experiment; |
| IgM-3     | Immunoglobulin M Dilution 3;  
Its concentration on the arrays is half of the concentration of control IgM-2;  
Used to assess the binding capacity of fluorescent-conjugated secondary incubation;  
Acts as a positive control when Cy3-anti human IgM secondary antibody is used in the experiment;  
Acts as a negative control when other secondary antibodies (e.g. anti-human IgG, anti-human IgA etc.) are used in the experiment; |
| IgM-4     | Immunoglobulin M Dilution 4;  
Its concentration on the arrays is half of the concentration of control IgM-3;  
Used to assess the binding capacity of fluorescent-conjugated secondary incubation;  
Acts as a positive control when Cy3-anti human IgM secondary antibody is used in the experiment;  
Acts as a negative control when other secondary antibodies (e.g. anti-human IgG, anti-human IgA etc.) are used in the experiment; |
| IgM-5     | Immunoglobulin M Dilution 5;  
Its concentration on the arrays is half of the concentration of control IgM-4;  
Used to assess the binding capacity of fluorescent-conjugated secondary incubation;  
Acts as a positive control when Cy3-anti human IgM secondary antibody is used in the experiment;  
Acts as a negative control when other secondary antibodies (e.g. anti-human IgG, anti-human IgA etc.) are used in the experiment; |
| IgM-6 | Immunoglobulin M Dilution 6;  
|       | Its concentration on the arrays is half of the concentration of control IgM-5;  
|       | Used to assess the binding capacity of fluorescent-conjugated secondary incubation;  
|       | Acts as a positive control when Cy3-anti human IgM secondary antibody is used in the experiment;  
|       | Acts as a negative control when other secondary antibodies (e.g. anti-human IgG, anti-human IgA etc.) are used in the experiment; |
| IgA-1 | Immunoglobulin A Dilution 1;  
|       | Used to assess the binding capacity of fluorescent-conjugated secondary incubation;  
|       | Acts as a positive control when Cy3-anti human IgA secondary antibody is used in the experiment;  
|       | Acts as a negative control when other secondary antibodies (e.g. anti-human IgG, anti-human IgM etc.) are used in the experiment; |
| IgA-2 | Immunoglobulin A Dilution 2;  
|       | Its concentration on the arrays is half of the concentration of control IgA-1;  
|       | Used to assess the binding capacity of fluorescent-conjugated secondary incubation;  
|       | Acts as a positive control when Cy3-anti human IgA secondary antibody is used in the experiment;  
|       | Acts as a negative control when other secondary antibodies (e.g. anti-human IgG, anti-human IgM etc.) are used in the experiment; |
| IgA-3 | Immunoglobulin A Dilution 3;  
|       | Its concentration on the arrays is half of the concentration of control IgA-2;  
|       | Used to assess the binding capacity of fluorescent-conjugated secondary incubation;  
|       | Acts as a positive control when Cy3-anti human IgA secondary antibody is used in the experiment;  
|       | Acts as a negative control when other secondary antibodies (e.g. anti-human IgG, anti-human IgM etc.) are used in the experiment; |
| IgA-4 | Immunoglobulin A Dilution 4;  
|       | Its concentration on the arrays is half of the concentration of control IgA-3;  
|       | Used to assess the binding capacity of fluorescent-conjugated secondary incubation;  
|       | Acts as a positive control when Cy3-anti human IgA secondary antibody is used in the experiment;  
|       | Acts as a negative control when other secondary antibodies (e.g. anti-human IgG, anti-human IgM etc.) are used in the experiment; |
| IgA-5 | Immunoglobulin A Dilution 5;  
|       | Its concentration on the arrays is half of the concentration of control IgA-4;  
|       | Used to assess the binding capacity of fluorescent-conjugated secondary incubation;  
|       | Acts as a positive control when Cy3-anti human IgA secondary antibody is used in the experiment;  
|       | Acts as a negative control when other secondary antibodies (e.g. anti-
| **IgA-6** | Immunoglobulin A Dilution 6;  
Its concentration on the arrays is half of the concentration of control IgA-5;  
Used to assess the binding capacity of fluorescent-conjugated secondary incubation;  
Acts as a positive control when Cy3-anti human IgA secondary antibody is used in the experiment;  
Acts as a negative control when other secondary antibodies (e.g. anti-human IgG, anti-human IgM etc.) are used in the experiment; |
| **ZZ_CON1** | Beta Galactosidase BCCP Myc;  
Acts predominantly as negative controls when performing the autoantibody assay but will be present signal intensities if any general reactivity towards BCCP is present;  
Acts as a positive control when performing the anti-c-myc assay on the arrays. |
| **ZZ_CON2** | BCCP Myc;  
Acts predominantly as negative controls when performing the autoantibody assay but will be present signal intensities if any general reactivity towards BCCP is present;  
Acts as a positive control when performing the anti-c-myc assay on the arrays. |
| **ZZ_CON3** | Beta Galactosidase BCCP (no Myc);  
Acts predominantly as negative controls when performing the autoantibody assay but will be present signal intensities if any general reactivity towards BCCP is present;  
Acts as a negative control when performing the anti-c-myc assay on the arrays. |
| **ZZ_CON4** | BCCP (No Myc);  
Acts predominantly as negative controls when performing the autoantibody assay but will be present signal intensities if any general reactivity towards BCCP is present;  
May also be used for downstream normalisation of signal intensities for any given study using the array.  
Acts as a positive control when performing the anti-c-myc assay on the arrays. |

LABORATORY RUN PROTOCOL

ASSAY 1: Autoantibody Assay

Reagent preparation 1 – Labelled anti-human IgG using PD10 desalting columns

Chemicals

<table>
<thead>
<tr>
<th>Item</th>
<th>Materials</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Polyclonal Rabbit Anti-Human IgG</td>
<td>Dako</td>
<td>A042301</td>
<td>4°C</td>
</tr>
<tr>
<td>2</td>
<td>Monoreactive Cy3-dye</td>
<td>GE Healthcare</td>
<td>GEH-PA23001</td>
<td>4°C</td>
</tr>
<tr>
<td>3</td>
<td>10X Phosphate Buffer Saline</td>
<td>BioSynTech</td>
<td>PB0344-1L</td>
<td>Room temperature</td>
</tr>
<tr>
<td>4</td>
<td>Glycine</td>
<td>Sigma Aldrich</td>
<td>G8898</td>
<td>Room temperature</td>
</tr>
<tr>
<td>5</td>
<td>18.2MΩcm Mili-Q Water</td>
<td>MiliPore</td>
<td>-</td>
<td>Room temperature</td>
</tr>
</tbody>
</table>

Consumables

<table>
<thead>
<tr>
<th>Item</th>
<th>Materials</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PD-10 columns</td>
<td>GE Healthcare</td>
<td>17-0851-01</td>
</tr>
<tr>
<td>2</td>
<td>0.5 ml non-translucent vials</td>
<td>Fisher Scientific</td>
<td>N/A</td>
</tr>
<tr>
<td>3</td>
<td>0.5 ml non-translucent screw cap</td>
<td>Fisher Scientific</td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>1.5 ml microcentrifuge tube</td>
<td>Various</td>
<td>N/A</td>
</tr>
<tr>
<td>5</td>
<td>15 ml centrifuge tubes</td>
<td>Various</td>
<td>N/A</td>
</tr>
<tr>
<td>6</td>
<td>Gloves</td>
<td>Various</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>10, 100 and 1000 µl tips</td>
<td>Various</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Aluminium foil</td>
<td>Various</td>
<td></td>
</tr>
</tbody>
</table>

Equipment

<table>
<thead>
<tr>
<th>Item</th>
<th>Materials</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NanoDrop 8000 Spectrophotometer</td>
<td>Thermo Scientific</td>
<td>ND-8000</td>
</tr>
<tr>
<td>2</td>
<td>Shaker</td>
<td>Jeiotech</td>
<td>SK-300</td>
</tr>
</tbody>
</table>
Precautions

1. Keep Polyclonal Rabbit Anti-Human IgG on ice where possible
2. Keep the reaction mixtures (Cy3 – anti-human IgG) dark (under aluminium foil) to reduce photo bleaching.
3. Do not allow the desalting columns to dry out at the surface as this hinders flow through to column.
4. Protein solutions should only be freeze-thawed once.

Procedure

A. Chemical preparation before starting

<table>
<thead>
<tr>
<th>Item</th>
<th>Stock Solution</th>
<th>Preparation</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1X PBS</td>
<td>Mix 100 ml of 10X PBS and 900 ml Mili-Q water</td>
<td>Room temperature</td>
</tr>
<tr>
<td>2</td>
<td>1M Glycine</td>
<td>Dissolve 7.51 g glycine in Mili-Q water to a final volume of 100 mL</td>
<td>4°C. Please use within 1 month</td>
</tr>
</tbody>
</table>

B. Measuring anti-human IgG concentration

1. Prepare anti-human IgG to determine the actual protein concentration and vortex to mix.
2. Open the spreadsheet ‘TEMPLATE for Cy3-IgG concentration calculator using NanoDrop’.
3. Open ‘Protein concentration’ program in NanoDrop software. Load 1.5 µl Mili-Q water on pedestal to wash. Wipe it off with KimWipes.
4. ‘Blank’ the NanoDrop using 1X PBS. Then clean with KimWipes.
5. Load 1.5 µl of anti-human IgG onto pedestal A and click ‘Measure’. Measure the anti-human IgG concentration in duplicate.
6. Save the data file in the designated files and make a note of the protein concentration and absorbance at 280 nm.
7. In the excel spreadsheet ‘Template for Cy3 IgG concentration calculator using Nanodrop’, enter the batch numbers of anti-human IgG and Cy3 dye that will be used in the labelling process.
8. Enter anti-human IgG stock concentration given on the vial in mg.ml⁻¹ into the spreadsheet.
9. Enter the protein concentration and 280 nm absorbance (A280 10mm) reading into the spreadsheet to calculate the IgG concentration giving 1 AU.

10. Save the spreadsheet in a folder with the name of the reagent batch.

11. **Acceptance criteria:** The relative standard deviation of the determination must be <5%.

12. Dilute down the IgG stock solution to 2 mg/ml by adding the required amount of 1X PBS which is given in the template.

13. Vortex thoroughly to mix after adding 1X PBS.

14. Keep the solution on ice.

**C. Antibody labelling**

1. Open the required number of Cy3 vials and place in a tube rack.

2. Add 1 ml of the 2mg/ml IgG stock solution to each of the dye vials.

3. Vortex thoroughly to dissolve the dye.

4. Incubate reaction vials at room temperature for 70 minutes under aluminium foil on the orbital shaker at speed 150. Keep the reaction mixtures under foil to reduce photo bleaching.

5. Quench the labelling reactions by adding 10 µl 1M glycine to each vial. Vortex to mix and incubate on ice for 10 minutes on the shaker.

   **Note:** PD-10 column purification can be carried out subsequently for each labelled antibody solution. Keep the remaining solutions on ice until processing.

6. Prepare five empty 1.5 ml Eppendorf tubes per PD 10 column to collect the fractions of eluate.

7. Prepare one PD-10 columns by equilibrating with 25 ml 1X PBS. Drain the liquid (25 ml of 1X PBS) to the surface of the columns.

   **Precaution:** Do not allow column from drying out.

8. Add 1ml of each labelling reaction to a column and allow immersing. Discard the flow through.

9. Add 1.5 ml of 1X PBS. Try to get a distinct clear band between the volumes.

10. Then further add 3.5 ml 1X PBS to flush out IgG-Cy3 from the PD-10 column. Get ready to collect the eluates in empty 1.5 ml Eppendorf tubes.
11. Start collecting the liquid as soon as it starts to become light pink in colour (Figure 1). Collect fraction of 0.5 ml volume in each of 1.5 ml Eppendorf tube. Stop collecting fractions once the pink colour gets darker.

![Free Cy3 – Unbind Cy3
Anti-human IgG - Cy3](image)

**Figure 1:** Distinct fraction between IgG-Cy3 and unbind Cy3.

12. Keep the fractions on ice until all the PD 10 columns have been eluted (If it is involved more than one PD 10 columns).

D. **Measuring labelled protein concentration**

1. Vortex the collected fractions to mix.
2. Open ‘Protein -Labeled’ program in NanoDrop software.
3. Use 1.5 µl of 1X PBS as a blank.
4. Then add 1.5 µl from tube 1 and click ‘Measure’. Wipe-off sample with KimWipes and repeat this process for all the fractions that have been collected.
5. Save the data file and make a note of the absorbance at 280 nm and 550 nm.
6. Open the spreadsheet ‘TEMPLATE for Cy3-IgG concentration calculator using NanoDrop’.
7. Enter the protein concentration and absorbance at the 280 nm and 550 nm into the spreadsheet to calculate the IgG concentration and labelling ratio.
8. If the Absorbance readings are outside the range of 0.2-1.2, adjust the dilutions so that the readings fall within this range and repeat the measurements.

The spreadsheet calculates the concentrations and labelling ratio according to the following equations:

a) **Antibody concentration calculation:**

\[
\text{Concentration Cy3-anti-IgG (mgml}^{-1}) = (A_{280} - (0.08 \times A_{550})) \times \text{Dilution} \times E
\]

Concentration giving 1AU at 280nm for anti-IgG = E

b) **Fluorophore concentration calculation:**
Concentration Cy3 (M) = $A_{552}/$Dilution/150000

Molar extinction coefficient at 552nm for Cy3 dye = 150000 M$^{-1}$cm$^{-1}$.

c) Recovery calculation:

Recovery (%) = \[\frac{\text{Volume of pooled IgG-Cy3 (}\mu\text{l}) \times \text{Average concentration (mgml}^{-1})}{\text{amount of antibody used in labeling reaction (}\mu\text{l}) \times \text{unlabeled antibody concentration (mgml}^{-1})}\]

d) Labeling ratio calculation:

Labeling ratio (nmole fluorophore (mg antibody)$^{-1}$) = Concentration Cy3 $\times 10^{6}$/ Concentration Cy3-anti-IgG

9. The template will help to determine which of the fractions from each column should be pooled together into a 15ml Falcon tube.

E. Combine fractions of Cy3 anti-human IgG

1. Pool the desired fractions into a 15ml Falcon Tube.
2. Perform another protein concentration determination as step 27 until 30 on the final pooled eluate sample and enter the readings into the template.
3. Enter the total volume of the pooled eluate sample into the template.
4. The template will determine the volume of 1X PBS to add to the pooled IgG-Cy3 to achieve the target protein concentration if needed.
5. Add the required volume of 1xPBS if necessary. Then perform a protein concentration determination on this normalized solution.
6. Enter the values for the normalized solution into the template.

**Notes:**
1. The actual protein concentration should be higher than the target concentration at this point and a series of dilutions will need to be performed to achieve the target concentration.

2. It is better to perform these dilutions in gradual steps to avoid overshooting the target and ending up with a labeled protein at a lower target value. Approximately three dilutions steps will need to be performed.

7. Enter the absorbance readings for the last dilution into the final product section of the template where the pass or fail criteria will be determined.
8. **Acceptance criteria:** The relative standard deviation of the determination must be <5%. The recovery must be between 70 % and 125 %.

9. Aliquot 220 µl of Cy3- anti IgG using a 1000 µl pipette into 0.5 ml non-translucent vials.

10. Tap the tubes to deposit the sample at the bottom of the tube then freeze at –20 °C.

### Reagent preparation 2 – Preparation of Serum Albumin Buffer (SAB)

#### Chemicals

<table>
<thead>
<tr>
<th>Item</th>
<th>Materials</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10X Phosphate Buffer Saline (PBS)</td>
<td>BioSynTech</td>
<td>PB0344-1L</td>
<td>Room temperature</td>
</tr>
<tr>
<td>2</td>
<td>Bovine Serum Albumin (BSA)</td>
<td>Sigma Aldrich</td>
<td>A3059-100G</td>
<td>4°C</td>
</tr>
<tr>
<td>3</td>
<td>Triton X100</td>
<td>Sigma Aldrich</td>
<td>T9284-100ML</td>
<td>Room temperature</td>
</tr>
<tr>
<td>4</td>
<td>18.2 MΩ-cm Mili-Q Water</td>
<td>MiliPore</td>
<td>-</td>
<td>Room temperature</td>
</tr>
</tbody>
</table>

#### Consumables

<table>
<thead>
<tr>
<th>Item</th>
<th>Materials</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Weighing boat</td>
<td>Various</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>5 mL tip</td>
<td>Eppendorf</td>
<td>0030000978</td>
</tr>
</tbody>
</table>

#### Equipment

<table>
<thead>
<tr>
<th>Item</th>
<th>Materials</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Laboratory Balancer</td>
<td>Mettler Toledo</td>
<td>JP1203C</td>
</tr>
<tr>
<td>2</td>
<td>Magnetic stirrer</td>
<td>Heidolph</td>
<td>505-30000-00</td>
</tr>
<tr>
<td>3</td>
<td>Magnetic stirring bar</td>
<td>Various</td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>Spatula</td>
<td>Various</td>
<td>N/A</td>
</tr>
<tr>
<td>5</td>
<td>Measuring Jug, 5 L</td>
<td>Various</td>
<td>N/A</td>
</tr>
<tr>
<td>6</td>
<td>5 mL pipette</td>
<td>Eppendorf</td>
<td>3120000070</td>
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</tbody>
</table>
### Procedure

**Serum Assay Buffer – 3 Litres**

<table>
<thead>
<tr>
<th>Item</th>
<th>Component</th>
<th>% (v/v; w/v)</th>
<th>Volume to add (mL)</th>
<th>Weight of component (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10X Phosphate Buffer Saline (PBS)</td>
<td>10%</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Triton X100</td>
<td>0.1%</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Bovine Serum Albumin (BSA)</td>
<td>*0.1%</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>18.2 MΩ-cm Mili-Q Water</td>
<td>Make up to a final volume of 3 L</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Proportion of weight to volume (w/v)

1. Collect 2.6 L of Mili-Q Water (18.2 MΩ-cm) in a clean 5 L measuring jug.
2. Add the 300 mL of 10X PBS into the 5 L measuring jug.
3. Then, add 3 mL of Triton X100.
4. Weigh out 3 g of BSA in a weighing boat using a smaller bench top balance.
5. Add the BSA to the PBS triton mixture (prepared from steps 1 to 3).
6. Put the measuring jug on magnetic stirrer plate and introduce magnetic stirring bar to the jug. Begin to stir the buffer. Continue stirring until the reagents are thoroughly mixed.
7. Add Mili-Q water to make up to a final volume of 3 L.
8. Store the buffer at 4°C.

### Assay run protocol

#### Chemicals

<table>
<thead>
<tr>
<th>Item</th>
<th>Materials</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Human serum test samples</td>
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<td>N/A</td>
<td>-20/-80°C</td>
</tr>
<tr>
<td>2</td>
<td>Human serum control</td>
<td>Sigma Aldrich</td>
<td>H4522-20ML</td>
<td>-20/-80°C</td>
</tr>
<tr>
<td>3</td>
<td>Cy3-rabbit anti-human IgG</td>
<td>In-house production</td>
<td>N/A</td>
<td>-20°C</td>
</tr>
<tr>
<td>4</td>
<td>18.2 MΩ-cm Mili-Q Water</td>
<td>MiliPore</td>
<td>N/A</td>
<td>Room temperature</td>
</tr>
<tr>
<td>5</td>
<td>Serum assay buffer (SAB)</td>
<td>In-house production</td>
<td>N/A</td>
<td>4°C</td>
</tr>
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</table>
## Consumables

<table>
<thead>
<tr>
<th>Item</th>
<th>Materials</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Quadriperm dishes</td>
<td>Greiner Bio-One</td>
<td>96077307</td>
</tr>
<tr>
<td>2</td>
<td>30 ml Pap jars</td>
<td>Evergreen Scientific</td>
<td>FIS#05-557-2</td>
</tr>
<tr>
<td>3</td>
<td>15 ml polypropylene centrifuge tubes</td>
<td>General</td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>5 ml tip</td>
<td>Eppendorf</td>
<td>30000978</td>
</tr>
<tr>
<td>5</td>
<td>200/1000 µl tip</td>
<td>General</td>
<td>N/A</td>
</tr>
<tr>
<td>6</td>
<td>10 µl tip</td>
<td>General</td>
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## Equipment

<table>
<thead>
<tr>
<th>Item</th>
<th>Materials</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Refrigerated incubator shaker</td>
<td>Jeiotech/Medline</td>
<td>SI-600R</td>
</tr>
<tr>
<td>2</td>
<td>Shaker</td>
<td>Jeiotech/Medline</td>
<td>SK-300</td>
</tr>
<tr>
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<td>20C Waterbath</td>
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</tr>
<tr>
<td>4</td>
<td>Vortex</td>
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</tr>
<tr>
<td>5</td>
<td>Microcentrifuge 13,000 g</td>
<td>General</td>
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</tr>
<tr>
<td>6</td>
<td>Microcentrifuge with MTP adapter</td>
<td>General</td>
<td>N/A</td>
</tr>
<tr>
<td>7</td>
<td>Wireless Barcode Scanner</td>
<td>General</td>
<td>N/A</td>
</tr>
<tr>
<td>8</td>
<td>Pap Jar racks (24 places)</td>
<td>General</td>
<td>N/A</td>
</tr>
<tr>
<td>9</td>
<td>15 ml tube rack</td>
<td>General</td>
<td>N/A</td>
</tr>
<tr>
<td>10</td>
<td>Polyacetyl rack</td>
<td>BRAND</td>
<td>BR471400</td>
</tr>
<tr>
<td>11</td>
<td>Polyacetyl trough</td>
<td>BRAND</td>
<td>BR471500</td>
</tr>
<tr>
<td>12</td>
<td>10-50 ml laboratory dispenser</td>
<td>Various</td>
<td>N/A</td>
</tr>
<tr>
<td>13</td>
<td>1-5 ml laboratory dispenser</td>
<td>Various</td>
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</tr>
<tr>
<td>14</td>
<td>Blunt forceps/spatula</td>
<td>General</td>
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</tr>
<tr>
<td>15</td>
<td>Volumetric flask glass 200 ml</td>
<td>General</td>
<td>N/A</td>
</tr>
<tr>
<td>16</td>
<td>5 ml Pipette</td>
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</tr>
<tr>
<td>17</td>
<td>200/1000 ul Pipette</td>
<td>General</td>
<td>N/A</td>
</tr>
<tr>
<td>18</td>
<td>10 ul Pipette</td>
<td>General</td>
<td>N/A</td>
</tr>
<tr>
<td>19</td>
<td>Lab Timer</td>
<td>General</td>
<td>N/A</td>
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<tr>
<td>20</td>
<td>MiliQ Water Purification System</td>
<td>General</td>
<td>N/A</td>
</tr>
<tr>
<td>21</td>
<td>Biological Safety Cabinet</td>
<td>General</td>
<td>N/A</td>
</tr>
<tr>
<td>22</td>
<td>Microarray Scanner</td>
<td>Agilent Technologies</td>
<td>G4600AD</td>
</tr>
</tbody>
</table>
**Before Starting**

1. Pour approximately 200 mL cold Serum Albumin Buffer (SAB) into a slide trough/dish and keep at 4°C until required.

2. Pour 2 litres of SAB into a 2 L Duran bottle and equilibrate to 20°C for 30 min in a designated circulating water bath. Fix a 50 mL laboratory dispenser to the bottle before use.

3. Pour 1 litre of SAB into a 1 L Duran bottle and equilibrate to 20°C for 30 min in a designated circulating water bath. Fix a 5 mL laboratory dispenser to the bottle before use.

4. Label the 15 mL Falcon tubes from number 1 up to 24 and place in order in a polystyrene tube rack.

5. Pipette 4.5 mL of SAB into each tube using the designated 5 mL Eppendorf laboratory dispenser.

6. Label the Quadriperm plates with consecutive numbers corresponding to the number of samples (up to 24) on the bottom of the plates.

7. Place 24 Pap jars into a suitable rack and label each tube with consecutive numbers corresponding to the number of samples (up to 24).

**Procedure**

**A. Samples Dilution Preparation**

1. Check each sample visually to ensure that each of the tubes has sufficient serum (22.5µL) for assay. Place in the Jeio Tech shaking incubator set at 20°C to thaw for 30 mins.

2. Then, vortex mix each sample for a count of three at full speed.

3. Centrifuge for 3 minutes at 13,000 g. Disinfect the centrifuge with 70% ethanol if spillages occur.
4. Dilute the serum/plasma in SAB buffer to provide the assay solutions. Take the first sample, call out the sample ID on the tube, and pipette 22.5 µL of the sample into 4.5 mL of SAB Buffer in tube number 1.

   Note: Teammate to write down the sample ID in batch record sheets. This step must be carried out in a Class II Biological Safety Cabinet. Be careful not to disturb the undiluted serum samples during movement.

5. Vortex to mix for a count of three at maximum speed. Place the vortexed tube in a different tube rack to avoid confusion with unused buffer tubes.

6. Repeat step 6 and 7 for the remaining samples.

   Note: Teammate to check that the correct samples are added to the correct tubes and mark the batch records accordingly.

B. Incubation with diluted samples

1. Take out the slide dish and rack containing 200 mL cold SAB.

2. Randomly pick 12 pap jars containing 2 protein array slides.

3. Take each slide in turn from their storage buffer by gripping the array between thumb and index finger at the labeled end of the slide.

4. Drain excess liquid from the slide by touching the edge of the array on the rim of the pap jar.

5. Lift the rack from the slide dish and place the slide in slot 2 with the barcoded side facing towards slot 1. Then place the rack back in the slide dish.

6. Add each slide in turn to the rack from left to right, making sure the slides are all in the same orientation.

7. When all the arrays have been added, gently shake the rack up and down five times to aid mixing at the slide buffer interface.

8. Put the lid on the slide dish and shake on an orbital shaker at 50 rpm, for 5 minutes.

   Note: Washing times exceed 5 minutes are not critical.

9. Pipette 4.0 mL of each diluted sample from the 15 mL Falcon tubes into their corresponding numbered Quadriperm chambers.
10. Place several layers of white laboratory tissue onto the bench surface and cover this with three layers of Kimwipe tissues.

11. When the slides have finished washing, remove each array in order from the rack. 
   **Note: Always place the rack back in the buffer between removing each array.**

12. Grip the slide at the labeled end between index finger and thumb and wipe the back of the slide once on the Kimwipe tissue. Then blot the long edge of the array three times on the wad of lint free tissue paper.

13. Place the slide immediately into Quadruperm chamber containing the diluted serum sample. Ensure that the array does not rest on the lugs at the bottom of the plate.

14. Set a timer to countdown for 2 hours after addition of the first array. Gently swirl the plate to cover the slide with incubation solution.

15. After addition of all slides, scan the barcode on each slides and it will automatically log into the relevant batch record.

16. Incubate all the Quadruperm dishes in the shaking incubator at 50 rpm, 20°C for 2 hours.
   **Note: Ensure that the dishes are kept horizontal at all times to prevent slopping of solutions between chambers. Handle the dishes very gently to prevent slopping or splashing of contents between chambers.**

C. Washing after Samples Incubation

1. Towards the end of the incubation period, fill 24 Pap jars with 30 mL of SAB.

2. When the incubation time has finished, remove each array from the Quadruperm dishes and wash each array individually in a Pap jar.

3. **Wash 1:** Use forceps or index finger to lift the array at the numbered side of the dish.

4. Grip the labeled end of the slide between finger and thumb and place the first slide into its respective number of Pap jar.
   **Note: Make sure that your gloves are scrupulously clean before handling arrays directly.**

5. Cap the Pap jar and invert four times before placing in order in the Pap jar rack on the shaker and shake at 50 rpm.
6. Start a timer to countdown 20 min after addition of the first array.

7. Process the remaining slides in order and place each in the Pap jar rack on the shaker whilst shaking at 50 rpm as they are prepared.

8. **Wash 2:** After the 20 minutes incubation has finished, take the first array and pour off the wash solution into an empty beaker then dispense another 30 mL of SAB into the tube at the back of the array. Invert the Pap jar four times and place in the Pap jar rack on the shaker at 50 rpm. Start the timer to countdown 20 min.

9. **Wash 3:** When the second wash step is nearly finished, prepare a slide staining box with rack and add 200 mL of SAB. When the second washing has finished, take the first Pap jar and pour off the buffer. Take the array between index finger and thumb and place in the slot 2 of the slide rack with the barcoded side facing towards slot 1. Place the rack back in the SAB.

10. Start the time to count down 20 min and add the remaining arrays sequentially until all slides have been transferred. Ensure the slides are all in the same orientation and order. Replace the slide rack in buffer between the additions of each array.

11. When all the arrays have been added, gently shake the slide rack up and down five times to aid mixing. Place the lidded box on a shaker for the remainder of the incubation time at 50 rpm.

**D. Incubation with Cy3-Anti-Human IgG**

1. Measure 200 mL of **SAB** at 20°C into a volumetric flask and add 200 µL of Cy3-antihuman IgG. Mix well by repeated inversion. Pour the solution into a fresh slide staining box (without the rack) and cover until required.

2. Place a wad of Kimwipe tissues on top of the working bench. Ensure that the benches do not become contaminated with buffer.

3. After the third wash is finished, lift the rack of arrays from the wash solution and place on the wad of Kimwipe tissues.

4. Bang the slide rack gently on the tissues five times to remove excess wash buffer. Immediately place the arrays in the Cy3-antihuman IgG solution.
5. Shake the rack up and down five times to help mixing of the probing solution at the surface of the arrays. Be careful not to shake the arrays out of the racks. Set a timer to countdown for 2 hours.

6. Lid the box and shake in the shaking incubator at 50 rpm, 20°C for the remainder of 2 hours incubation time.

E. Washing after Cy3-Anti-Human IgG incubation

1. Towards the end of the incubation period, fill a slide staining box with 200 mL of fresh SAB buffer (20°C).

2. **Wash 1:** When the incubation has finished, lift the slide rack from its incubation solution and place into the fresh SAB wash solution.

3. Shake the rack gently up and down five times. Replace the lid and shake for 5 min at 50 rpm at room temperature.

4. **Wash 2:** After wash 1 has finished, lift the slide rack out of the dish and pour off the buffer into a beaker. Pour in 200 mL of fresh SAB buffer (20°C).

5. Shake the rack gently up and down five times. Replace the lid and shake for 5 min at 50 rpm at room temperature.

6. **Wash 3:** After wash 2 has finished, lift the slide rack out of the dish and pour off the buffer into a beaker. Pour in 200 mL of fresh SAB buffer (20°C).

7. Shake the rack gently up and down five times then replace the lid and shake for 5 min at 50 rpm at room temperature.

8. When the third wash has finished, lift the slide rack out of the dish and pour off the SAB. Fill the box with high purity water.

9. Place the slide rack in the water and shake gently up and down five times. Then pour off the high purity water. Repeat this step three times to ensure the buffer components are washed away from the slide rack and arrays.

10. Place a wad of Kimwipe tissue on the clean bench and also in the clean and dry staining box.

11. Remove the slide rack from the dish and bang gently five times on the wad of Kimwipe tissue to remove excess water.
12. Place the slide rack in the fresh staining box on top of the Kimwipe tissues.

F. Drying down the slides

1. Lid the box and dry the arrays by centrifugation for 4 minutes at 400 g.

   *Note: Add a balancing box if necessary.*

G. Scanning

1. Place the slide in the slide holder with the barcoded side facing upward. Close and lock the cassette lid.

2. Place the slide holder into the Agilent slide carousel.

3. Scan the slides at 10µm resolution, 16-bit.
ASSAY 2: Protein-protein Interaction

Consumables

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Procedure

A. Samples preparation and Cy3 conjugation

1. Thaw the sample on ice.

2. Quantify the sample using Nanodrop, to measure protein concentration.

3. Add the protein solution (1 ml) to the vial containing coupling buffer and mix thoroughly by gentle vortexing or by manually inverting the capped tube 10 times.

   **Caution:** This dye is intensely coloured and very reactive. Care should be exercised when handling the dye vial to avoid staining clothing, skin and other items.
4. Transfer the entire volume of protein and coupling buffer to the Cy3 vial, cap it and mix thoroughly. Care should be taken to prevent foaming of the protein solution. Incubate the solution at room temperature for 1 hour and 30 mins and shake at 100 rpm. Cover the tube with aluminium foil to avoid dye bleaching.

5. While the labelling reaction is incubating, decant the buffer from the top of the column and add 13 ml fresh elution buffer.

6. Carefully transfer approx. 1 ml of protein-labelling mixture into the column and allow the solution to enter the resin bed.

7. Add 2.0 ml of elution buffer. As this volume of buffer moves through the column, a faster moving pink band of labelled protein will separate from the unconjugated dye.

8. Add an additional 2.5 ml of elution buffer to the column and collect the faster moving pink band in clean tubes as it elutes from the column. The labelled protein should be entirely eluted by 2.5 ml of buffer.


B. Blocking LifterSlip

1. Immerse the LifterSlips in LifterSlip blocking buffer (See Appendix A) and incubate for 2 hours at room temperature.

2. Continue blocking step for overnight in 4°C.

3. Wash with LifterSlip blocking buffer three times, 5 minutes at RT.

C. Blocking IMMUNOME slides

1. Remove IMMUNOME microarray slides from -20°C storage, and rinse in 3 ml Wash Buffer (See Appendix A) for each slide, 5 minutes.

2. Block slides in CT100plus blocking buffer (See Appendix A) for 1 hour.

3. Wash slide three times, 5 minutes in Wash buffer at RT.
D. Incubating with the labelled samples

1. Prepare the labelled samples at concentrations of 40, 200 or 1000 ug/ml in **PPI buffer** (See Appendix A).
2. Incubate each slide with each labelled sample for 2 hours, on ice at 50 RPM.
3. Wash slides 2x 10 minutes in PPI buffer on ice at 50 RPM.
4. Wash slides 1x 10 minutes in PPI buffer (no Triton X-100) on ice at 50 RPM.
5. Dry slide at 400 x g for 4 minutes at RT

E. Scanning

1. Place the slide in the slide holder with the barcoded side facing upward. Close and lock the cassette lid.
2. Place the slide holder into the Agilent slide carousel.
3. Scan the slides at 10µm resolution, 16-bit.

**Appendix A: Buffer recipes and reagents**

- **Wash buffer**: 1x PBS and 0.2% Tween-20.
- **CT100plus blocking buffer**: 25 mM HEPES (pH 7.46), 50 mM KCl, 20% glycerol, 50 mM biotin, 0.1% Tween-20.
- **Lifter slip blocking buffer**: 0.1% BSA in 1x PBS.
- **PPI buffer**: 10mM Tris-HCl (pH 7.4), 0.2 mM EDTA, 100 mM potassium glutamate, 0.2%Triton X-100
ASSAY 3: Citrullination

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### Procedure

A. Blocking IMMUNOME slides

1. Remove IMMUNOME microarray slides from -20°C storage, and rinse in 3 ml Wash Buffer (See Appendix A) for each slide, 5 minutes.
2. Block slides in CT100plus blocking buffer (See Appendix A) for 1 hour.
3. Wash slide three times, 5 minutes in Wash buffer at RT.

B. Citrullinating IMMUNOME slides

1. Add 3 ml of 1 μg/ml PAD2/PAD4 (in Citrullination buffer (See Appendix A)) to the slides and cover the apparatus with aluminium foil and incubate for 3 hours at 37°C at 50 RPM.
2. Wash the slides in 3 ml of Wash buffer for 3 x 5 minutes at RT.

C. Incubation with diluted samples

1. Check that each sample has sufficient serum/plasma volume for the assay. Place the samples on the shaking incubator for 30 minutes.
2. Vortex each sample for a count of three at full speed.
3. Centrifuge the samples for 3 minutes at 13,000 x g.
4. Add 3 ml of diluted patient samples (1: and incubate for 2 hours at 20°C.
5. Wash the slides in 3 ml of Wash buffer for 5 minutes at 20°C. Repeat this step twice.

D. Incubation with Cy3-anti-human IgG
1. Add 3 ml of 1 μg/ml Cy3-labelled anti-human IgG antibody (in Wash buffer) to all slides, and incubate for 2 hours at 20°C.
2. Wash the slides in 3 ml of Wash buffer for 5 minutes at 20°C. Repeat this step twice.
3. Wash slides in 3 ml of 1XPBS for 5 minutes at 20°C.
4. Dry slides at 400 x g for 4 minutes at RT.

E. Scanning
1. Place the slide in the slide holder with the barcoded side facing upward. Close and lock the cassette lid.
2. Place the slide holder into the Agilent slide carousel.
3. Scan the slides at 10μm resolution, 16-bit.

Appendix A: Buffer recipes and reagents
- Washing buffer: 1x PBS and 0.2% Tween-20.
- CT100plus blocking buffer: 25 mM HEPES, 50 mM KCl, 20% glycerol, 50 mM biotin, 0.1% Tween-20.
- Citrullination buffer: 25 mM HEPES, 5 mM CaCl₂, 150 mM NaCl, 0.2% Tween-20.
4 BIOINFORMATICS PROTOCOL

Background

Functional protein microarrays differ in many respects from DNA or RNA microarrays. Unlike DNA microarrays, functional protein microarrays often aim to discover global interactions of a single probe (protein) in a one colour-channel, which results in a relatively small selection of specific proteins showing strong signals for a given sample. Furthermore, the identity of these proteins often varies between samples (Duarte, J., et. al., 2013). This is proven by numerous mass spectrometry-based proteomic experiments which have reported high variations of protein intensities between samples in the serum proteome. Therefore, conventional microarray approaches to biomarker discovery such as data normalisation and the downstream analyses which follow are not applicable to functional protein microarrays.

Image Analysis

The aim of an image analysis is to evaluate the amount of autoantibody present in the serum sample by measuring the median intensities of all the pixels within each probed spot. A raw .tiff format image file is generated for each slide, i.e. each sample. Automatic extraction and quantification of each spot on the array are performed using the GenePix Pro 7 software (Molecular Devices) which outputs the statistics for each probed spot on the array. This includes the mean and median of the pixel intensities within a spot along with its local background. The following is a brief description of how an image analysis is performed for the Sengenics Immunome Protein Array using GenePix Pro 7 software.

1. **Loading .tiff images** – Array images are automatically barcoded and saved in .tiff format after scanning. .tiff images are loaded into GenePix Pro 7 software to be analysed.

   **Note: GenePix only reads image data in 16-bit gray scale**
2. **Automatic Spot Detection** - A GAL (GenePix Array List) file for each array is generated to aid image analysis. Each GAL file will contain a list of proteins with respect to their positions on each array. Please note GAL files are grid files specific to GenePix software and may not be compatible with any other software. Loading the GAL file onto GenePix Pro 7 software will automatically generate grids on the array slide. Grids are then automatically aligned on each spot for data extraction.

3. **Data Extraction** - Data output consist of probe name, protein name, position, median Relative Fluorescence Unit (RFU) and background RFU for each spot. Results are generated and saved in .gpr format.

![Figure B: Loading a GAL file onto GenePix Pro 7 Software](image)

![Figure C: Data output from GenePix Pro 7 Software](image)
Data Handling and pre-processing

For each slide, proteins and control probes are spotted in quadruplicate - 4 arrays on each slide. All steps are represented in flowchart (Figure E). The following steps were performed to verify the quality of the protein array data before proceeding with data analysis:

**Step 1:** Calculate net intensities for each spot by subtracting background signal intensities from the foreground signal intensities of each spot. For each spot, the background signal intensity was calculated using a circular region with three times the diameter of the spot, centered on the spot. Figure D is a visual representation of how this calculation was carried out (Molecular Devices).

**Step 2:**
Remove replica spots with RFU ≤ 0.

**Step 3:**
Zero net intensities if only 1 replica spot remaining.

**Step 4:**
Calculating percentage of coefficient of variant (CV%) to determine the variations between the replica spots on each slide.

$$CV\% = \frac{\text{S.D.}}{\text{Mean}} \times 100\%$$  

Equation 1

![Figure D. Visual representation of local background calculation methods. The spot coloured in red represents the spot of interest.](image-url)
Flag a set of replica spots with only 2 or less replica/s remaining and CV% > 20% as “High CV”. The mean RFU of these replica spots (i.e. proteins) will be excluded from the downstream analysis.

For proteins/controls with a CV% > 20% and with 3 or more replica spots remaining, the replica spots which result in this high CV% value were filtered out. This was done by calculating the standard deviation between the median value of the net intensities and individual net intensities for each set of replica spots. The spot with the highest standard deviation was removed. CV% values were re-calculated and the process repeated.

**Step 5:**

Calculating the mean of the net intensities for the remaining replica spots.

**Step 6:**

Inspecting signal intensities of two positive controls: IgG and Cy3-BSA.

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**IgG Serial Dilution – Quality Control**

The IgG dilution series acts as a positive control for assessing the binding capacity of fluorescent-conjugated secondary incubation. Accurate serial dilution quantification is used as a benchmark for ensuring that labelling efficiency and spot detection pass quality control thresholds. The experimental ratios for the dilution series of IgGs are as follows:

\[
IgG1 : IgG2 : IgG3 : IgG4 : IgG5 : IgG6 \]

\[
x : 0.5x : 0.25x : 0.125x : 0.0625x : 0.03125x
\]

Equation 2
where $x$ is the initial concentration (i.e. EFU) of IgG spot. Figure F shows an image of all the replica IgG spots’ serial dilution on the Immunome slide.

![Image of IgG spots serial dilution](image)

**Figure F: SENGENICS-IMMUNOME™ Protein Array quadruplicate images for IgG control spots showing serial dilution (Left to right).**

The methods used to ensure the same amount of secondary antibody used in the experiment are as follow:

1. Plots of mean net intensities of IgG 1 – 6 spots
2. Plot of CV% for IgG 1 – 6 and calculate the average IgG CV% across all samples. Threshold of CV% is 15%.
3. Calculates the average CV% between the IgG serial dilution across all samples and the experimental dilution.

**Cy3-BSA Analysis – Quality Control**

Cy3-BSA controls act as positive controls for each array on the slide. Cy3-BSA 1 – 23 markers are present on each slide and their concentration are kept constant throughout the experiment.

![Image of Cy3-BSA control spots](image)

**Figure G: SENGENICS-IMMUNOME™ Protein Array replica images for all 23 Cy3BSA control spots showing. For slide orientation purpose, Cy3BSA 1 to 16 are spotted in replicates of 5 and Cy3BSA 17 to 23 are spotted in quadruplicate.**
Methods used to ensure consistency of Cy3-BSA controls probes are as follows:

1. Plot of mean net intensities for Cy3-BSA 1 -23.
2. Plot of CV% for Cy3-BSA 1 – 23 and calculate the average Cy3-BSA CV% across all samples. Threshold of CV% is 15%.
3. Calculate the average CV% between 23 Cy3-BSA spots within each slide across all samples.

Data Normalisation

Composite normalisation of data using both quantile-based and total intensity-based modules (Duarte, J. et al., 2013;2018) This method assumes that different samples share a common underlying distribution of their control probes while taking into account the potential existence of flagged spots within them. The Immunome array uses Cy3-labelled biotinylated BSA (Cy3-BSA) replicates as the positive control spots across slides. Hence it is considered as a housekeeping probe for normalisation of signal intensities for any given study.

The quantile module adopts the algorithm described by Bolstad et al., 2003. This reorganisation enables the detection and handling of outliers or flagged spots in any of the Cy3BSA control probes. A total intensity-based module was then implemented to obtain a scaling factor for each sample. This method assumes that post-normalisation, the positive controls should have a common total intensity value across all samples (Causton H.C. et al., 2004) This composite method aims to normalise the protein array data from variations in their measurements whilst preserving the targeted biological activity across samples. The steps are as follows:

Quantile-Based Normalisation of all cy3BSA across all samples

(i=spot number and j=sample number)

1. Load all Cy3-BSA across all samples, j, into an i X j matrix X
2. Sort spot intensities in each column j of X to get Xsort
3. Take the mean across each row i of Xsort to get < Xi >
Intensity-Based Normalisation

1. Calculate sum of the mean across each row i, ∑<i>Xi</i>
2. For each sample, k, calculate the sum of all Cy3-BSA controls, ∑Xk
3. For each sample, k,

$$\text{Scaling factor (k)} = \frac{\sum <X_i>}{\sum X_k}$$

Biomarker Discovery – A Recommended Approach

The recommended approach for identification and ranking of biomarkers for the Immunome Protein Array studies us by using a penetrance-based fold change (pFC) method. This measures the likelihood that a given raw fold change is significant, thus increasing the relevance and reliability of results. Biomarker discovery from protein arrays is markedly different from that cDNA, RNA, and DNA arrays. The frequency of any given single biomarker is far lower in a cohort study when looking at autoantibody biomarkers compared to mRNA or DNA biomarkers.

High concentrations of an arrayed protein may occasionally give a false positive signal in serology assays because of concentration-driven, non-selective binding of an immunoglobulin to the target. This can arise theoretically due to an avidity effect: weak, non-specific immunoglobulin binding sites on a specific protein becoming coupled across multiple neighboring protein molecules via an antibody as a result of the high density of immobilized protein, thus making the protein appear to be highly antigenic. Whenever this phenomenon occurs, it would be expected to be observed in the healthy control samples and will give rise to high intensity signals and/or signals that are close to saturation on the arrays. In Immunome, proteins such as RBPJ and IGHG1 show consistently high signal intensities across all samples.

For this reason, given a large sample number (i.e. 100 – 200 samples) and availability of sample cohort, a penetrance-based fold change (pFC) analysis method is implemented for the identification of highly expressed proteins in each case sample. This method will remove any false positive signals from the data by setting a protein-specific threshold (i.e.
background threshold). This threshold is calculated on a per-protein basis via the mean intensity of the signal for each specific protein measured for healthy control samples. A step-by-step description of this method is as flowchart (Figure H) and described as below:
Step 1:
Individual fold changes for both case and control are calculated by dividing the RFU value for each protein in each sample, H, by the mean of the RFU values of each protein across all the control samples (i.e. background threshold).

\[
\text{Individual FC} = \frac{H_{\text{Case or Control}}}{\mu(H_{\text{Control}})}
\]

Equation 1

Step 2:
For proteins with individual fold change of less than 2-fold above the background threshold, their signal intensities (RFU) are replaced with zeroes.

Step 3:
Penetrance frequency (number of case and control samples with individual fold changes ≥ 2-fold) for both case \(\text{Frequency}_\text{case}\) is determined for each protein.

\[
\text{Frequency}_\text{case} = n(\text{Individual FC (Case)} \geq 2)
\]

\[
\text{Frequency}_\text{control} = n(\text{Individual FC (Control)} \geq 2)
\]

Equation 2
Equation 3

Step 4:
Penetrance Fold Changes for both case and control groups are calculated for each protein.

\[
\text{Penetrance Fold Change}_\text{case} = \frac{\mu(H_{\text{Case \{\}|}})}{\mu(H_{\text{Control}})}
\]

Equation 4

\(H_{\text{case \{\}|}} = H_{\text{case with FC Case \geq 2 fold}}\)

Putative biomarkers are identified and ranked according to the following criteria:

- \(\text{Penetrance Fold Change}_\text{case} \geq 2\).
- \% \text{Frequency}_\text{case} \geq 10\
- \% \text{Frequency}_\text{control} \leq 10\%
Immunome Data Processing Pipeline

Immunome pipeline scripts are in R language (https://www.r-project.org/). Installation of certain pre-requisite packages is required prior to execution of scripts. Commands to install required packages are described in scripts. Scripts are available upon request.
6. **References**


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12. Miscellaneous – This Agreement shall be governed by and construed in accordance with the laws England and subject to the exclusive jurisdiction of the English Courts without regard to any conflict of laws principles that would require the application of the laws of a different jurisdiction. Customer and Sengenics expressly agree that the United Nations Convention on Contracts for the International Sale of Goods will not apply to these Terms or to any transaction.
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14 Publications
14.1 The Parties may publish at any symposia, national, international or regional professional meeting or in any journal, thesis, dissertation, newspaper or otherwise of its own choosing, the findings, methods and results derived from the Project, but always subject to due observance of this Clause 14.

14.2 The Party intending to make the publication ("the Publishing Party") shall provide the other Party any proposed publication or presentation in advance of the submission of such
proposed publication or presentation to a journal, editor, or other third party. The other Party having received such proposed publication (“the Receiving Party”) shall have thirty (30) days to identify any Confidential Information or potentially patentable subject matters which need protection and provide written comments to the Publishing Party. If no objection is made to the proposed publication or presentation within the stipulated time, the Publishing Party shall be free to proceed with the publication or presentation. In accordance to scientific custom, each Party shall, in any publications it makes in relation to the methods, results and findings of the Project, acknowledge the other Party’s contributions to the Project.

14.3 Confidential Information identified by the Receiving Party which is governed by Clause 15 shall be deleted from the proposed publication or presentation unless the Receiving Party agrees to treat the Confidential Information as patentable information, as set forth in Clause 14.4.

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15.3 Sengenics may request that both Parties file one or more patent applications on Joint IP. If Sengenics makes such a request and the Collaborating Party agrees with such a request, both Parties shall:
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(b) seek and maintain the strongest and broadest patent claims practicable in the best interest of the Parties.

15.4 Sengenics shall have ownership of commercialisation rights to the Projects IP.

15.5 Sengenics shall have, in consideration for payment of royalties from profit to, exclusive rights to manufacture and market globally any products or methods from the Project IP resulting from the Project.

15.6 If Sengenics declines its rights by notice in writing to other Party to commercialise the Project FIP, the other Party shall then have full rights to commercialise the Project FIP.