i-Ome Discovery – The Power of Precision

Who Are Sengenics?

Sengenics supports precision medicine by providing physiologically relevant disease associated data used to guide clinical research. Our solutions enable the discovery and validation of autoantibody biomarker signatures across a wide range of diseases for patient stratification, drug response prediction, and development of companion diagnostics. Our groundbreaking KREX® protein folding technology, developed at University of Cambridge, is applied to the manufacture of high-quality functional protein microarrays with the highest sensitivity and reproducibility on the market. Our new i-Ome Discovery protein microarray has over 1800 carefully selected autoantigens run with dual isotypes to maximize discovery, sensitivity, and specificity. Content has been carefully curated for its biological relevance to diseases such as cancer, neurodegenerative, infectious, and autoimmune. Recognizing the added value of multiple antibody isotype screening, Sengenics is one of the only companies that offers dual isotype screening as a standard assay, providing more than 3600 data points for every sample. Sengenics' expert scientists simplify the discovery process by guiding best-practice experimental design, execution, and interpretation of i-Ome Discovery data, guiding the process from inception to biological meaning. Along with the i-Ome Discovery array, Sengenics has also developed i-Ome AI open-source software that empowers Sengenics users to make independent data discoveries and share these discoveries with stunning publication-ready plots and graphs. Sengenics has more than 15 years of experience constructing protein microarrays and aiding researchers across various disciplines. The i-Ome Discovery protein microarray was devised and constructed as a team-effort involving our own experts, expert academicians, and partners from the pharmaceutical industry.

How Do We Succeed in Biomarker Discovery?

Autoantibodies are ideal biomarkers. They are early, direct manifestations of disease, produced at the first signs of a threat to health. Autoantibodies are convenient, easy to collect from simple blood draws, are stable for lengthy periods, and can be stored frozen for years. Due to their high specificity, antibody and autoantibody testing have long been standards in identifying infectious and autoimmune diseases. In many autoimmune diseases, autoantibodies appear years prior to any symptom or official diagnosis, an indication that autoantibodies are valuable predictors of future illness (*Bizzaro, 2007*). More recently, autoantibody signatures have shown great promise in the early detection and prognosis of complex diseases such as Parkinson's, Alzheimer's and various cancers (*Anuar et al., 2022; DeMarshall et al., 2016; Patel et al., 2022*). Further, focusing on autoantibody screening expedites biomarker discovery because disease-associated autoantigens represent only a small portion of the proteome, allowing for a very small number of physiologically relevant, antigenic proteins to represent a large number of functional, disease-associated pathways (*Sexauer et al., 2022; Williams & Wolin, 2021; Zaenker et al., 2016*). With five different antibody isotypes (*Table 1*), each with distinct activation and signaling properties, screening multiple simultaneously improves both sensitivity and specificity of disease detection and outcome prediction compared with single isotype screening and screening via other methodologies. Lastly, autoantibody immunoprofiling uncovers directly pathogenic and/or disease-reporting antigens and autoantigens, highlighting protein pathways directly implicated in disease, and alerting researchers to potential new druggable pathways. Altogether, autoantibody immunoprofiling is a cost-effective, low noise approach to biomarker discovery and improved mechanistic understanding of disease.

Antibody	Subclasses	Primary Function	Serum Level (g/L)
IgA	IgA_{1-2}	Pathogen neutralization, anti-inflammatory, mucosal	0.6 - 4
IgG	IgG_{1-4}	Circulating and tissue immunity	7 - 15
IgD	None	Upper aerodigestive immunity, B-cell development, immune regulation	0 - 0.14
IgE	None	Tumor surveillance, anti-venom defense, anti-parasitic defense, type I hypersensitivity	Trace
IgM	None	Immune surveillance, acute response (esp. agglutination, complement activation)	0.6 - 3

Table 1. Antibody Isotype Primary Functions with Adult Serum Reference Ranges.

What is i-Ome Discovery?

Sengenics i-Ome Discovery is our flagship discovery functional protein microarray specifically designed to produce biologically meaningful results. The culmination of decades of experience and collaboration with industry and academic leaders, i-Ome Discovery represents the most comprehensive disease-focused protein microarray currently available.

Curated content focused on known and potential autoantigens helps keep the array small, facilitating biomarker discovery with significantly lower false discovery rates and smaller sample sizes than other technologies.

Among the more than 1800 proteins on the array, there is high diversity, including representation of key kinases, transcription factors, immune signaling proteins, cancer-relevant proteins, neuronal proteins, and endocrine system proteins. Kinases such as AKT and MAPKs have been instrumental in developing over a dozen anti-cancer drugs. Transcription factors such as ATF4 participate in oxidative and endoplasmic reticulum stress, as well as tumor progression and cell death. Proinflammatory proteins such as STAT1, TNFα, and IL-18 ease the study of infectious and chronic inflammatory diseases. A multitude of antigens known to be targeted during immunodeficiency are also present. Tumor-specific and tumor-associated antigens (including various cancer-testis antigens) help enable discovery of cancer-relevant autoantibodies. Finally, neuronal proteins such as enolase, synuclein, and neurofilament light chain assist in the study of neurological disorders, while endocrine proteins such as thyroid hormone receptor and adrenergic receptors can aid in the study of endocrine disorders.

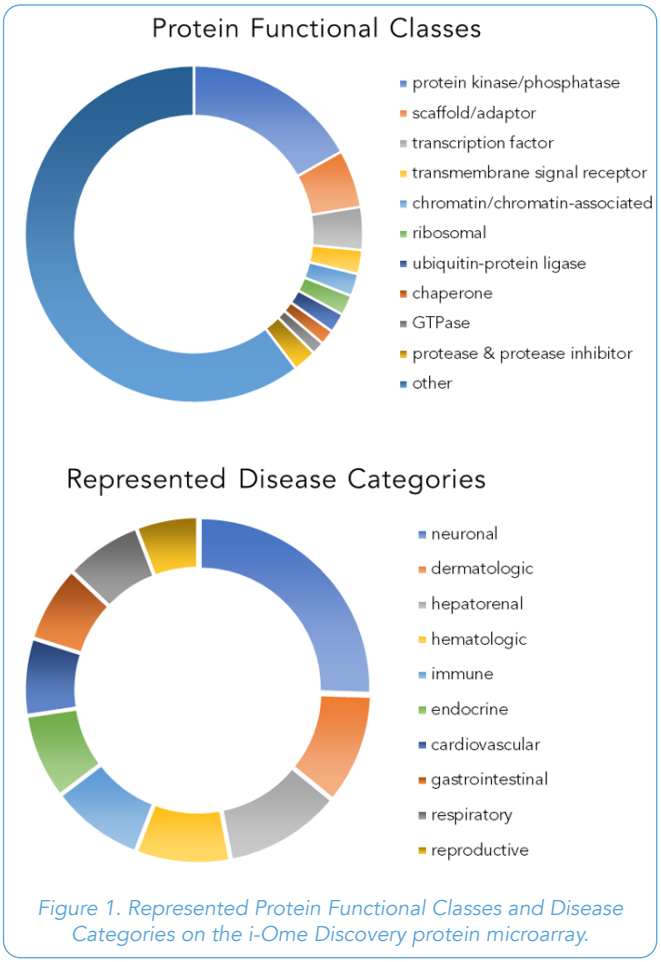

Figure 1. Represented Protein Functional Classes and Disease Categories on the i-Ome Discovery protein microarray.

The i-Ome Discovery protein microarray consists of valuable disease-centric proteins that help researchers concentrate their efforts on only the most relevant targets. The functional and disease categories represented by i-Ome Discovery are illustrated in *Figure 1*. The full list of proteins can be downloaded here. Array content includes a high number of intracellular and intranuclear proteins, as well as cell surface and extracellular proteins. Together, this provides excellent coverage to detect autoantibodies directly involved in disease pathogenesis as well as in reporting on disease states.

Biomarker	Name
SPATA19*	Spermatogenesis-associated protein 19
TSPY3*	Cancer Testis Antigen 78/Testis Specific Protein Y-Linked 3
GLS2	Glutaminase 2
TCEA2*	Transcription Elongation Factor A2/TFIIS/Testis-Specific SII gene
TSGA10*	Testis-specific gene protein 10/Cancer Testis Antigen 79
HMGN5	High Mobility Group Nucleosome Binding Domain 5/NSBP1
LUZP4*	Leucine Zipper Protein 4/Cancer Testis Antigen 28
HDAC4	Histone Deacetylase 4
SPACA3*	Sperm Acrosome membrane-associated protein 3/Cancer Testis Antigen 54
IMPDH1	Inosine Monophosphate Dehydrogenase 1/LCA11
TXN2	Thioredoxin 2/MT-TRX/COXPD29
TFG	Trafficking from ER to Golgi Regulator/TRKT3 Oncogene/TRK-Fused Gene Protein
PPP2R1A	Protein Phosphatase 2 Scaffold Subunit alpha/Serine Threonine Protein Phosphatase 2A

*Table 2. Autoantibodies correlating with poor survival in NSCLC patients following lung resection (AUC 0.918). *Cancer Testis Antigens.*

The proteins used in i-Ome Discovery have been highly informative for outcome prediction, patient stratification and biomarker discovery, illustrating the merits of screening autoantigenic proteins in critical physiological pathways. Further, i-Ome Discovery is built using the patented KREX® technology that ensures only full length correctly folded proteins form the array. This technology results in highly specific antibody antigen binding and incredible array reproducibility. For example, researchers utilizing this technology uncovered a set of 13 autoantibodies among non-small cell lung cancer patients predictive of poor survival following surgical resection (*Table 2*). The panel showed high clinical sensitivity and specificity in both the training and validation cohorts indicating the strong reliability and reproducibility of the array (*Patel et al., 2022*). In another example, the array capably identified distinct repertoires of autoantibodies between rheumatoid arthritis patients with and without anti-citrullinated protein antibodies (ACPA+ or ACPA-) providing a data driven means of patient stratification (*Figure 2*) (*Cunningham et al., 2023*). And for biomarker discovery, 6 novel autoantibodies associated with Alzheimer's Disease were discovered, including

autoantibodies to SOX15, a transcription factor typically involved in embryogenesis (*Wang et al., 2020*). While these assays were single isotype detection assays, focused on patient IgG antibodies, the i-Ome Discovery microarray detects two isotypes simultaneously for greater detail and depth of understanding disease.

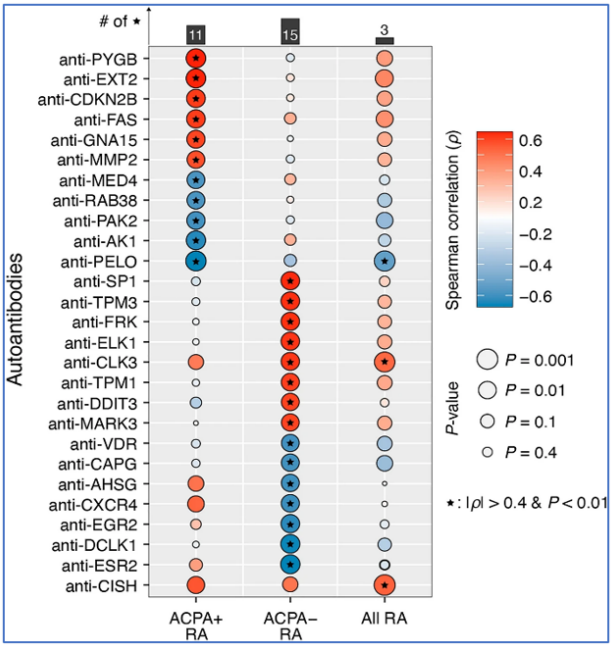

Figure 2. Top autoantibodies detected in ACPA+ and ACPA- Rheumatoid Arthritis patients. Creative Commons — Attribution 4.0 International — CC BY 4.0

Sengenics' i-Ome discovery is combined with dual isotype detection as a standard service. When using single isotype detection, anti-IgG secondary antibodies are used to detect patient antibodies bound to autoantigenic proteins on the array. However, since serum encompasses multiple antibody isotypes, and subclasses, using a single isotype array detection results in the loss of a considerable amount of valuable information. In a dual color detection array, two different isotype specific secondary antibodies are utilized for visualization, maximizing the value of the run (for more information see white paper Isotypes Diving Deeper into 230523 (*sengenics.com*)).

A standard Sengenics protein microarray assay includes IgG and IgA secondary antibodies (*Figure 3*). Coupled with more than 1800 proteins, screening for two isotypes in one assay produces over 3600 data points. Sengenics also has the capability to run IgM. Running multiple isotypes improves coverage across biologically relevant antibodies that can appear at different disease stages and under different conditions, consequently empowering biomarker discovery and resulting in greater clinical sensitivity and specificity than a single isotype detection assay.

The i-Ome Discovery protein microarray covers a broad range of protein functional classes and disease categories making it suitable for both targeted and untargeted approaches to biomarker discovery. Our

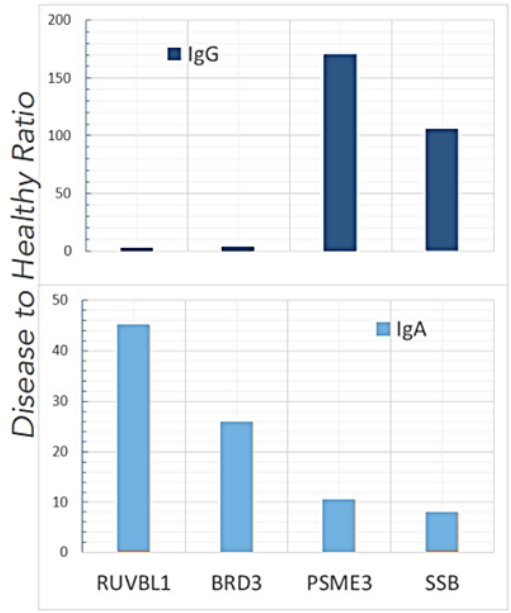

Figure 5. Pooled sera from autoimmune disease patients and healthy controls compared using the Sengenics IgG/IgA Dual Color Assay shows unique biomarkers of each isotype. The IgG and IgA isotypes identify different antigens, improving the level of detection.

immunologists continually add new curated content to our design database, ensuring that each updated version of i-Ome Discovery keeps pace with industry changes.

What makes Sengenics' i-Ome Discovery protein microarrays exceptionally reproducible?

The first functional protein microarrays were run in the early 2000's. However, several factors limited widespread usage. Cloning libraries were in their infancy, high throughput protein purification techniques were lacking, methods to print functional proteins needed development, and methods were needed to control inter- and intra- slide variability (*Bertone & Snyder, 2005*). Different and inconsistent methodologies led to the propensity for inaccurate and irreproducible data.

In a functional protein microarray, thousands of proteins are printed onto a glass slide with functionalized surface chemistry. The sample is most often patient serum and plasma, although urine, cerebrospinal fluid, other biofluids, tissue homogenate and culture supernatant have been used. Sample antibodies bind to their cognate antigen on the array and are detected via indirect immunofluorescence (*Figure 3*). The quality of the data is directly linked to antibody binding specificity which is influenced by several factors such as antigen shape, charge, temperature, and other denaturing factors. In fact, antibody binding specificity

is highly dependent on antigen shape (watch the video *https://youtu.be/y3nLPMpGMpc*). Ninety percent of antibodies recognize the discontinuous epitopes formed by protein folding (*Van Regenmortel, 1996*). Proteins must retain their 3D conformational shape throughout the duration of processing, or the specific binding epitope will be lost. A poorly envisioned slide microenvironment can cause protein deformation, flattening or sliding into neighboring spots, further confounding array results, and if not controlled, protein adherence to the slide can mask the epitope. High quality data relies on preserving the shape and availability of the antigen epitopes. If these are not addressed, then the rate of false positives and false negatives increase from non-specific binding and unavailable epitopes. Sengenics protein microarray production technology tackles these concerns.

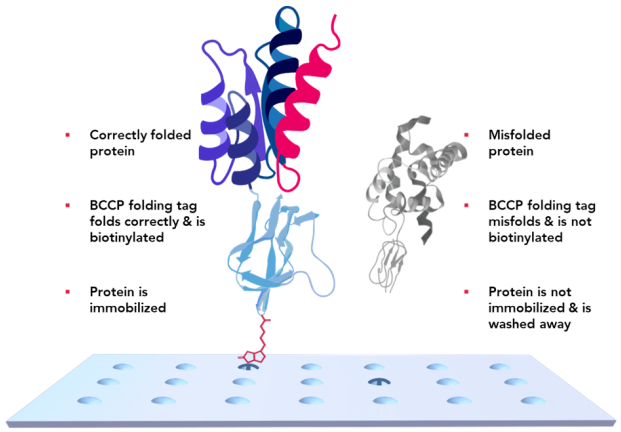

Figure 4. KREX® Protein Folding Technology

Sengenics KREX® protein folding technology is designed to ensure the protein array consists of full length, properly folded proteins with intact discontinuous epitopes. In this patented technology, a biotin carboxyl carrier protein (BCCP) is coded in-frame with each array protein as a folding marker. A misfolded or fragmented protein causes BCCP misfolding, masking its biotin site and preventing it from binding to the streptavidin coated array surface (*Figure 4*). The BCCP can be attached to either the N- or C- terminus of the protein during recombination. The biotin promotes consistent protein adherence to the slide, tethering the protein to the streptavidin in the same orientation and with exposed epitopes with each printing. The slides are hydrogel coated, forming an aqueous environment that preserves protein tertiary structure while preventing denaturing and flattening. Another important feature of the KREX technology is the use of insect cell lines as the host for producing the proteins. Insect cell lines offer numerous advantages over traditional yeast or bacterial lines including higher recombination success rates and preservation of post translational modification sites (*Beeton-Kempen et al., 2014; Blackburn et al., 2012*). Further, each protein is printed in quadruplicate which helps ensure statistical accuracy. Altogether, KREX® technology advances the quality and reproducibility of functional protein microarrays by maintaining protein tertiary structure. This is illustrated empirically in *Figure 6* in a comparison of two different protein microarray slides treated with the same serum sample.

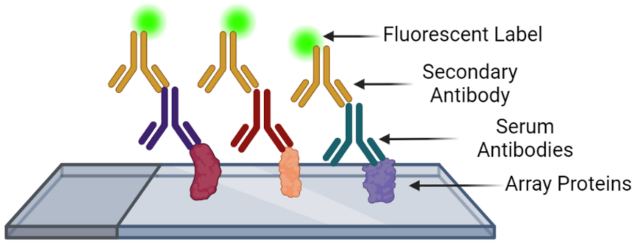

Figure 5. The set up of a Functional Protein Microarray.

How did we build the protein list?

The proteins on the chip have been carefully selected by our expert immunologists in collaboration with industry leaders. Specifically, they cover a wide range of known clinically relevant autoantigens, as well as proteins exhibiting key characteristics of the human autoantigenome. In addition, antigens have been selected to cover a broad range of protein functional classes and subcellular locations, as well as for excellent representation of key disease-associated molecular pathways, including coverage of potential drug targets. The array is enriched for nuclear and intracellular proteins with the ability to report on mechanisms of cell death. Kinases and nucleotide binding proteins facilitate detection of abnormalities in intracellular signaling and nucleotide binding. Kinases are often mutated in cancer and are among the easier proteins to target with drugs. Enriched content includes metabolic, growth, proliferation, and apoptosis related proteins, all of which contribute to health and disease. Known surface and extracellular autoantigens are also well represented. This can be confirmed with functional enrichment analysis using FunRich (*www.funrich.org*) and STRING (*www.string-db.org*). GeneCards with GeneAnalytics can be used to explore tissue distribution and disease contribution of the i-Ome Discovery array proteins (*www.genecards.org*).

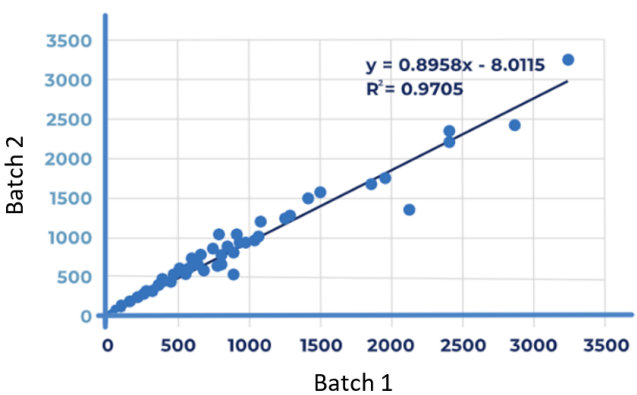

Figure 6 Batch to batch consistency. Control proteins across batches label with R2>95.

Functional annotation can be done with PANTHER (*www.pantherdb.org*). GSEA can be used to examine other important proteins and pathways across different disease conditions from the proteins present on the array (*www.gsea-msigdb.org/gsea/index.jsp*).

How do I interpret the data?

Sengenics staffs a bioinformatics team with 30 plus years' experience working with protein microarrays and protein microarray data. Starting with understanding the customer research objectives and available resources, the bioinformatics team works directly with customers, walking them through a very comprehensive best-practice program to facilitate optimal study design (including appropriate sample sizes) and demonstrate the Sengenics analytical pipeline as well as expected outputs from every study. Depending on objectives, sample sizes can range from <20 subjects in simple pilot studies to >150 subjects in larger biomarker discovery and validation efforts. In most cases, 100 µl serum samples are needed from each subject for the study. Upon receipt of samples, they are checked and diluted prior to assay on the i-Ome Discovery platform. Raw data undergo extensive sample- and assay-level quality control to ensure that only the highest-quality data are taken forward for downstream analysis. Data processing procedures are illustrated in *Figure 7*.

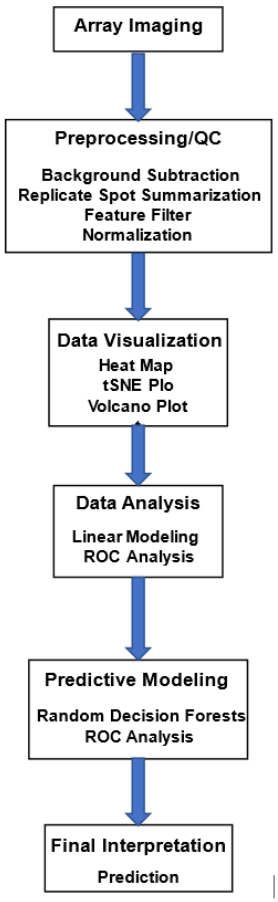

Figure 7 Data Processing and Interpretation Workflow. Note that some tests and algorithms will require a larger number of subjects than others.

Each array contains four replicates of each protein. Greater detail on the techniques used to process and analyze the data can be found in our White Paper, "*Bioinformatics for Immunoprofiling with Protein Microarrays.*"

The bioinformatics team evaluates and prepares the data for visualization and interpretation. Linear modeling is applied to the data to evaluate significant differences among features (protein hits) across different groups. The team has expertise with unsupervised clustering (machine learning) and random decision forest tests to be applied to larger datasets for biomarker discovery, response prediction and target identification studies. The team has extensive knowledge of these techniques and will aid throughout the process. The team uses tools like FunRich, STRING and GeneCards (see above) to evaluate the data for functional differences across groups. Using these tools coupled with our deep understanding of the proteins present on i-Ome Discovery, our team will share insights into the pathways influenced by the conditions of each group within the experiment. This includes target enrichment, network analysis, pathway enrichment and pathway activation state. Lastly, the team developed a user-friendly, open-source data analysis platform called i-Ome AI. I-Ome AI is an intuitive, easy to use, AI driven bioinformatics platform that automatically processes Sengenics's protein microarray data and provides the end user with meaningful, detailed reports on the results.

Concluding Remarks

Under disease and distressed conditions, aberrant protein behavior occurs that the humoral immune system detects and marks with autoantibodies. Consequently, autoantibodies are direct early biomarkers of potential health issues. The i-Ome Discovery functional protein microarray has been meticulously designed to effectively capture these biomarker signatures. The array includes over 1800 thoughtfully curated, physiologically relevant, often autoantigenic proteins that participate in key pathways implicated in cellular dysfunction and disease. Data reproducibility is ensured with the KREX® protein folding technology. The creation of this microarray was a collaborative endeavor that drew upon the expertise of industry professionals, our team of biologists, and our bioinformatics experts. Leveraging our team's extensive experience and deep understanding of proteomics, we are dedicated to guiding our customers through the entire process, from the initial idea to the acquisition of robust data and the generation of meaningful and interpretable results.

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