Suspension Bead Arrays: Powerful Tools for Biomarker Discovery and Validation

Introduction

For several years, significant efforts have been made to understand the human proteome, with the ultimate goal of identifying protein biomarkers that may be used to detect and characterize disease states and enable targeted drug development. While these studies have often used mass spectrometry/gel electrophoresis approaches, protein affinity arrays have now emerged as very useful biomarker discovery and validation tools for large-scale proteome analysis of large numbers of samples.

While planar arrays can interrogate biological fluids for many proteins simultaneously, suspension bead arrays have several advantages. For example, they enable fluid-phase kinetics, which are faster than the solid-phase kinetics of planar arrays. Suspension bead arrays also provide higher reproducibility, because hundreds of beads are measured for each analyte.[1] The flexibility of bead-based systems enable creation of different arrays on demand by mixing only the desired antibodies with the beads, rather than having to fabricate new planar arrays for each application. Bead-based arrays also lend themselves very well to automation and can provide greater dynamic range than planar arrays.[2] To be of the most use, these arrays require a source of human proteins and antibodies that represent the breadth and depth of the human proteome.

The Human Protein Atlas

Several efforts have been initiated to provide affinity reagents for human proteome targets, including ProteomeBinder, SH2 Consortium, NCI affinity capture project, and Human Protein Atlas.[3] This publicly accessible atlas (www.proteinatlas.org) is an outcome of the Swedish Human Proteome Resource program that runs a high-throughput process that starts from bioinformatic human antigen selection based on low similarity to other protein coding genes and proceeds to cloning, production, and purification of the antigens. These are then used to generate validated antibodies. Polyclonal antibodies have been produced against more than 80% of human protein encoding genes.[4] These antibodies have been used in a multitude of studies that employed Luminex® xMAP® Technology to construct suspension bead arrays for use in protein biomarker discovery and validation.

Luminex xMAP Technology

Luminex internally dyes bead sets with precise concentrations of fluorescent dyes, resulting in 500 distinctly colored bead sets (**Figure 1**). Each bead set can be coupled with reagents for specific bioassays such as antigens, antibodies, or oligonucleotides. Any combination of bead sets can be used in a single assay, enabling multiplex detection of up to 500 analytes from a single reaction volume. The bead mixture is incubated with the sample and detected on a Luminex instrument using a reporter dye to quantify the amount of bound analyte.

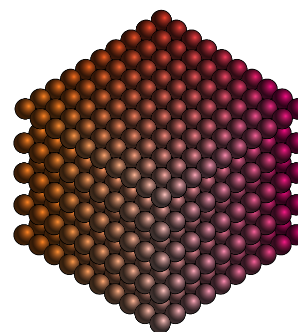

Figure 1. Luminex internally color-codes microspheres with precise concentrations of various florescent dyes, yielding up to 500 distinctly colored bead sets.

Depending upon the instrument used, up to 500 bead types can be used in each well of a 96- or 384-well plate, thus generating a high-throughput assessment of a large number of protein or oligonucleotide targets. This microsphere based "liquid array" system for measuring analytes is unique in its ability to provide both high-throughput and high-content data, and researchers are able to easily scale the number of analytes measured as well as customize both the assays and types of applications.

xMAP Technology lends itself well to antibody array studies, as the workflow is simple and does not require purification, and picomolar detection levels and dynamic ranges of more than three orders of magnitude have been demonstrated.[5] As a result, xMAP suspension bead arrays have been used in a wide variety of biomarker studies.

Serum and Plasma Profiling

An early study at the KTH-Royal Institute of Technology in Sweden utilized xMAP suspension bead arrays to establish their utility for profiling serum that was first reacted with biotin to enable a subsequent labeling reaction.[5] Beads were coupled to 27 different monospecific antibodies (msAbs) that targeted 20 different serum proteins. Following incubation of the biotinylated serum with the beads, phycoerythrin-labeled streptavidin was added to enable detection of the bound serum proteins (**Figure 2**). Analysis of 222 serum samples from twins revealed a higher correlation than samples selected randomly. Importantly, these samples were prepared, labeled and analyzed in less than two working days. A follow-up study at the same institution entailed a systematic comparison of serum and protein profiles using these suspension bead arrays.[6] No significant differences were observed between the two sample types for almost 80% of the 174 antibodies tested. However, plasma offered greater biological variability and more opportunity for biomarker discovery.

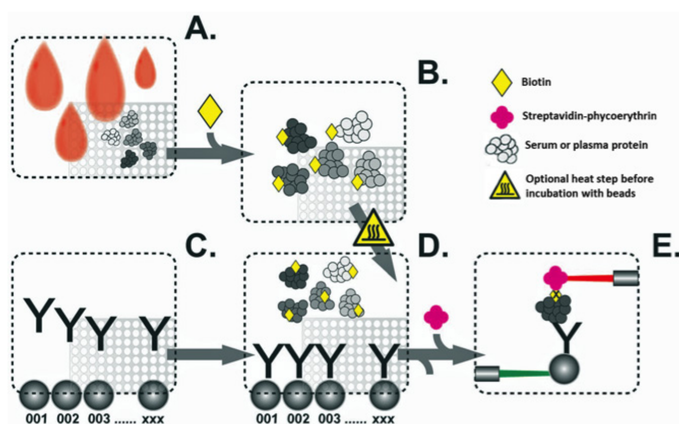

Figure 2. Experimental procedure of antibody suspension bead arrays using biotinylated samples. The process starts with the distribution of samples into microtiter plates according to a defined, randomized sample positioning (A). The protein content of samples is then labeled with biotin B) and antibodies are coupled onto beads with distinct color codes to create a suspension bead array (C). Beads and samples are combined for incubation after the samples have been heat treated in assay buffer (heat treating is an optional step used to expose epitopes (D). Proteins that have not been captured by antibodies are removed and fluorescent (phycoerythrin) streptavidin is added for detection (D). Each bead type is then identified via a red laser, and the emitted reporter fluorescence of each bead of the same type is determined using a green laser. The mean fluorescence intensity for each bead type is a measure of the presence and amount of a specific protein present in the sample that has reacted with its corresponding antibody, attached to the beads (E). Adapted from Darmanis et al (2013), doi:10.1371/journal.pone.0081712.g003. This work is licensed under a Creative Commons Attribution 2.0 Generic License.

A subsequent large-scale comparison of planar and suspension bead arrays for serum profiling was also performed at the KTH-Royal Institute of Technology. Using 68 monospecific antibodies from the Human Protein Atlas project to analyze serum from 12 donors, the two platforms provided correlating results; particularly for medium to high abundance serum proteins.[7] Suspension bead arrays did provide slightly more sensitivity when using antibodies with high intensity signals.

In an extension of the biotinylated plasma protein approach, a study was performed using heat-induced epitope retrieval to enhance detection of proteins such as those comprising the complement system.[3] Heating the biotinylated plasma at 56 or 72°C gave the optimal results, with 40% of the antibody-defined protein profiles exhibiting positive intensity changes, using 96 monospecific antibodies and 84 plasma samples. Applying this heat-induced epitope approach, a study of plasma from prostate patients identified several proteins with differential profiles between control and prostate plasma. Utilizing xMAP beads and the Luminex® 100/200 analyzer, approximately 4 x 1000 samples with 100 antibodies, or ~40,000 assays, could be completed in a working day. This approach thus provides a noticeable advantage in sample throughput over planar arrays.[3]

A longitudinal study of serum from twins was conducted to understand sources of variation in suspension bead array profiling.[8] The majority of the experimental variation was attributed to experimental sources. Attention to sources of experimental variation, including complexity and composition of the serum samples and variations in biotin modification of samples can help reduce the proportion of such experimental variation, as well as reduce the sample size required to detect epidemiological effects of interest.[8]

Multiple Sclerosis as an Autoimmune Disease

In addition to enabling efficient profiling of plasma and serum proteins, suspension bead arrays can be used to profile the human autoantibody repertoire by binding protein fragments to the xMAP beads. In a study of multiple sclerosis (MS) at the KTH-Royal Institute of Technology, an undirected and systematic approach using protein epitope signature tags (PrESTs) from the Human Protein Atlas was used to profile IgG populations from 90 multiple sclerosis subjects.[9] A total of 2000 antigens showed reactivity for 11,520 PrESts attached to planar arrays, and 64% of these were found only in single individuals. Reactivity distributions were used to select 384 antigens identified as potentially interesting candidates, and these were verified using both planar and suspension bead arrays. The bead arrays were further employed to interrogate more samples (376), resulting in the identification of a set of 51 antigens, which displayed differences in recognition for different disease subtypes, including primary progressive MS (PPMS), secondary-progressive MS (SPMS), relapsing-remitting MS (RRMS), and several others.[9]

A parallel study by the same research group was undertaken to profile the cerebral spinal fluid (CSF) of subjects with MS, in an effort to develop biomarkers that could discriminate between disease subtypes.[10] A set of 43 proteins targeted by 101 antibodies (primarily from the Human Protein Atlas) bound to xMAP beads was chosen based on potential disease relevance from the literature or involvement in inflammatory mechanisms. Initial efforts identified an effect of total protein amount in the sample on systematic intensity differences. This was addressed by the addition of BSA and rabbit IgG to the sample biotinylation reaction. Discriminating potential with altered intensity levels between disease sub-types was observed for two proteins, growth associated protein 43 (GAP43), and serpin peptidase inhibitor clade A (SERPINA3). Samples from SPMS subjects were characterized by significantly lower levels of GAP43, relative to samples from subjects with early stages of MS and the control group of other neurological diseases. In contrast,

SERPINA3 exhibited higher levels in all MS patients compared to controls. This assay procedure thus offers promise for broad-scale protein profiling of CSF within neurological disorders.[10]

Most recently, the true power of xMAP suspension bead arrays was demonstrated in a large scale effort to profile plasma, CSF and brain tissue from subjects with MS.[4] Using antibodies generated primarily within the Human Atlas Project, an initial screening was performed using 4595 antibodies specific for 3450 genes. A collection of bead sets containing up to 384 antibodies each was used to cover the full range of 4595 antibodies. These bead sets were then used to screen plasma from 22 MS subjects. A set of 375 antibodies with discrimination power was selected from this initial screening and used for targeted analysis in a set of 172 plasma samples. Finally, 101 antibodies representing 41 genes were used to interrogate 443 plasma and 572 CSF samples. The result was a set of five proteins with intensity profiles that differentiated MS subtypes in both sample types: interferon regulatory factor 8 (IRF8), interleukin 7 (IL7), methyltransferase like 14 (METTl14), solute carrier family 30 (zinc transporter), member 7 (SLC30A7), and GAP43.[4] Expression and association analysis using immunofluorescence with these antibodies was subsequently conducted on human post-mortem brain tissue. The results revealed that neurons in the proximity of lesions were stained with antibodies for IRF8, IL7, and METTL14. The protein targets of these antibodies are thus candidates for further studies on the etiology of MS in brain tissue.[4]

Cancer Biomarkers

The lack of specificity and sensitivity of the prostate serum antigen (PSA) test for prostate cancer is widely recognized, as it can be used primarily to identify patients with advanced disease. Proteomics-based biomarker discovery using bead suspension arrays may enable identification of candidates with greater utility in the diagnosis of prostate cancer. In a study that was mentioned previously, xMAP bead suspension arrays were used to identify the protein carnosine dipeptidase 1 (CNDP1) as a potential marker for aggressive prostate cancer.[3] A follow-on study utilized several additional monospecific polyclonal antibodies for distinct epitopes that were identified using 15-mer peptides bound to xMAP beads, and that spanned CNDP1 antigens 1 and 2.[11, 12] These additional antibodies were used in a sandwich assay with unlabeled plasma from subjects with prostate cancer, as well as healthy controls. The results confirmed that CNDP1 levels decreased in subjects with prostate cancer. They also revealed that CNDP1 levels were different in subjects with lymph node metastasis, versus subjects that did not exhibit metastasis. These sandwich assays allowed improved differentiation of tumor N stages versus clinical tPSA, except when classifying T or M stages.[11]

Well-differentiated small intestine neuroendocrine tumors (WD-SI-NETs) are most often diagnosed after metastasis, hampering efforts to provide curative treatment. Earlier detection and improved monitoring of the disease are therefore required to enable control of this disease. A study has been conducted using xMAP bead suspension arrays using antibodies from the Human Protein Atlas that targeted 124 proteins.[13] Using biotinylated serum samples from subjects with untreated primary WD-SI-NETs, lymph node metastases and liver metastases, a set of 20 antibodies was initially identified that could have value for differentiating disease stages. Using an independent subject cohort, classification accuracy up to 85% was obtained with various subsets of these 20 antibodies, tested in respective group comparisons (**Figure 3**). Nine protein targets were identified as having profiles that significantly contributed to tumor classification: insulin-like growth factor binding protein 2 (IGFBP2), insulin growth factor 1 (IGF1), SH3KBP1 binding protein 1 (SHKBP1), v-ets avian erythroblastosis virus E26 oncogene homolog 1 (ETS1), interleukin 1 alpha (IL1α), syntaxin 2 (STX2), mastermind-like 3 (MAML3), early growth response protein 3 (EGR3), and X-linked inhibitor of apoptosis (XIAP).[13]

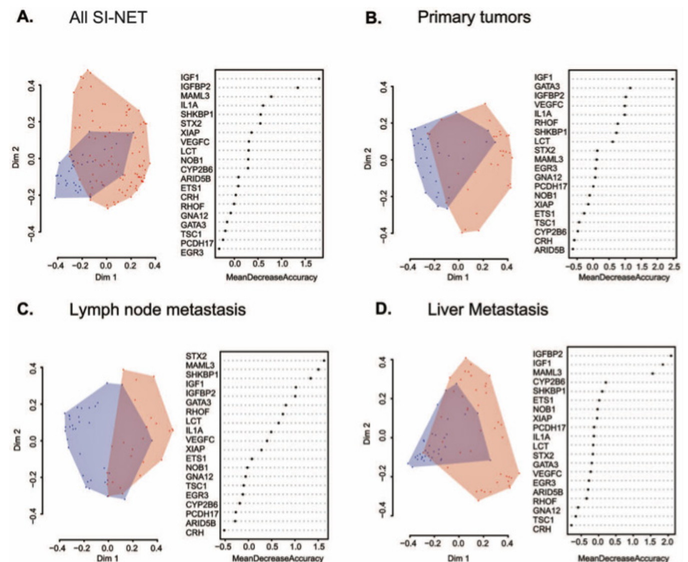

Figure 3. Multivariate classification of different individual groups. Distribution of each individual sample belonging to the healthy control group (blue circles) and all SI-NET (small intestine neuroendocrine tumor) patients (A), patients with primary tumors (B), lymph node metastasis (C), and liver metastasis (D). Axes values correspond to the two-dimensional projection of the proximity matrices generated for each pairwise comparison using RF. For each group, the relative importance of each protein used in the multivariate classification is shown. doi:10.1371/journal.pone.0081712.g003.
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Biomarkers for Other Diseases

The availability of reliable and sensitive biomarkers for renal impairment could enable detection of early signs of kidney toxicity and efficient monitoring of disease. Antibody bead suspension arrays based on xMAP Technology have been used to profile plasma from four types of kidney disorders: glomerulonephritis, diabetic nephropathy, obstructive uropathy, and analgesic abuse.[14] Biotinylated plasma samples were interrogated with bead arrays containing 129 polyclonal antibodies for 94 targeted proteins. The protein targets were chosen using pathway analysis and proteomics and gene expression data, selecting proteins known to be associated with disease, as well as proteins expressed in normal adult kidney. Analysis of 200 samples from clinical-associated cases and controls detected human fibulin-1 at significantly higher levels in subjects with glomerulonephritis (GN) compared to controls. This protein was also found at elevated levels in samples from the other

renal disorders. A verification study was also done with 16 samples from a separate cohort, including eight subjects with GN and eight healthy controls, using all four fibulin antibodies that were included in the initial study of 200 samples. The results confirmed the utility of fibulin-1 as a discriminating biomarker for GN.[14]

Biomarker discovery for rare diseases can be hampered by the relative scarcity of samples. A recent study of muscular dystrophy leveraged sample collection efforts by the BIO-NMD consortium at four clinical sites in Europe. A total of 345 blood samples were surveyed for 315 unique proteins targeted by 384 antibodies generated by the Human Protein Atlas, using xMAP bead suspension arrays.[15] Eleven proteins were identified that were associated with muscular dystrophy. Four of these proteins were elevated in blood from muscular dystrophy patients: mitochondrial malate dehydrogenase 2 (MDH2) and electron transfer flavoprotein A (ETFA), as well as carbonic anhydrase III (CA3) and myosin light chain 3 (MYL3), both of which are specifically expressed in slow-twitch muscle fibers. In addition, nine protein profiles were elicited which correlated with disease progression and severity. These biomarkers may enable the development of new clinical tools for management of dystrophinopathies.[15]

While osteoarthritis (OA) is one of the most disabling disease processes and the most common rheumatic disease, diagnostic methods are very limited, and no medications are yet available to stop its characteristic cartilage degeneration. Biomarkers useful for the early diagnosis, prognosis, and therapeutic monitoring of OA would therefore be of great interest to the medical community. A novel biomarker study of OA has been undertaken that utilized both planar arrays and bead suspension arrays to define autoantibody profiles that could be of diagnostic value.[16] The alterations involved in joint degeneration can be specifically detected by the immune system, even though OA is not an autoimmune disease. The result is a humoral immune response that produces immunoglobulins (autoantibodies) against the antigens created by the OA disease process.

An initial, untargeted screen for autoantibodies was performed on a set of 62 samples using 3840 PrESts from the Human Protein Atlas spotted on planar antigen arrays. From this screen, 373 antigens were selected for validation on bead-based arrays. A parallel targeted approach was done using NAPPA (nucleic acid programmable protein arrays) planar arrays, which utilizes cDNA encoded antigens and an in vitro expression system. In this case, the array consisted of 80 preselected proteins. Altogether, this dual-array approach identified nine (xMAP arrays) and seven (NAPPA) autoantibody targets.[16]

While antigen-antibody immune complexes are useful in suspension bead arrays for biomarker discovery, they can also stimulate a range of effector functions in vivo, including activation of the complement system. In any given autoimmune or infectious disease, these functions can therefore possibly add another important dimension for categorizing antigens or classifying clinical samples. In fact, an xMAP bead array-based assay for determining multiplexed antigen-specific antibody levels in parallel with their complement activation properties has been developed.[17] The assay utilized native and citrullinated peptide antigens bound to the beads to interrogate the levels of IgG, IgM and IgA autoantibodies, as well as their complement activating properties, in serum samples from 41 rheumatoid arthritis subjects and 40 controls. The degree of complement activation by all three complement activation pathways was measured via the deposition of C3 fragments from serum samples (**Figure 4**).

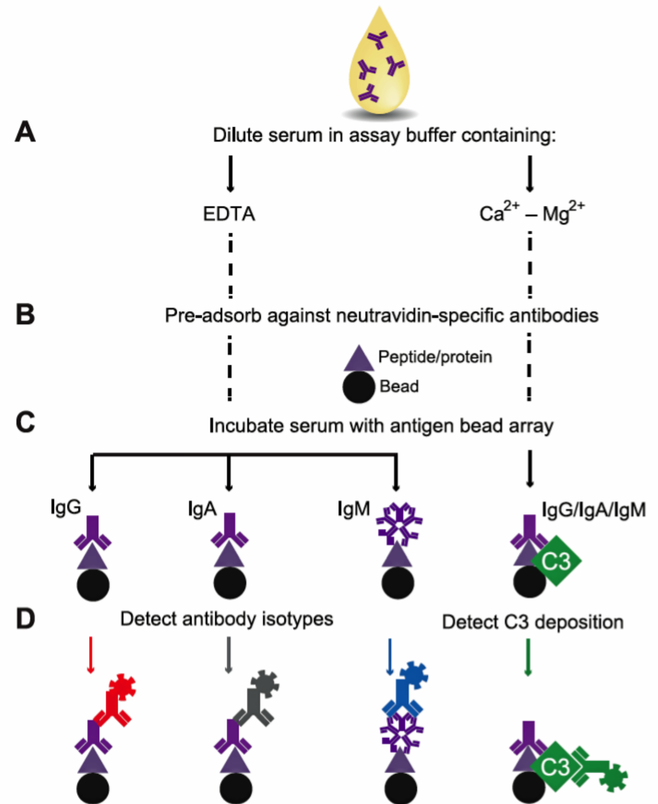

Figure 4. Schematic representation of assay workflow for multiplexed determination of antigen-specific antibody levels in parallel with their properties for complement activation. A) Serum samples are diluted 1:10, either in a Ca2+-Mg2+ containing assay buffer for detection of complement activation, or in an EDTA containing assay buffer for antibody detection. B) The samples are pre-adsorbed against neutravidin-specific antibodies. C) A mixture of beads coupled to various antigens is distributed into a 384-well plate and the pre-adsorbed samples are added to the bead array. D) Complement activation driven C3 deposition and different antibody isotypes are detected in parallel with fluorescently labeled secondary antibodies dispensed into individual wells of each quadrant of the 384-well plate. doi:10.1371/journal.pone.0096403.g001.
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Significantly higher IgG reactivity against the citrullinated fibrinogen β and filaggrin peptides was observed. In addition, an IgA reactivity exclusive for citrullinated fibrinogen β peptide and C3 deposition in rheumatoid arthritis patients was also detected. Rheumatoid factors interfere with the complement activation measurement, potentially resulting in false positives. This suspension bead array-based workflow can be used to study other autoimmune or infectious diseases in which the presence of complement-activating antibodies can provide diagnostic significance. This approach thus represents a significant development for analysis of autoantibody- or virus-associated immune pathologies.[17]

Luminex xMAP Technology

Luminex provides the only flexible and open multiplexing technology that is used by several market leaders to provide assays for both gene and protein expression. Unlike conventional technologies that can only measure one or a few biomarkers, researchers have the capability to easily scale up or down the number of biomarkers measured and to customize assays. xMAP Technology combines advanced fluidics, optics, and digital signal processing with proprietary microsphere technology. Featuring a flexible, open-architecture design, xMAP Technology can be configured to perform a wide variety of bioassays quickly, cost-effectively and accurately. Focused, flexible multiplexing of 1 to 500 analytes meets the needs of a wide variety of applications, including the construction of large suspension bead arrays for biomarker discovery and validation. An ever-expanding menu of assays for other applications is also available from Luminex and its commercial partners.

Partial List of Institutions using Luminex xMAP Suspension Bead Arrays for Biomarker Research

Department of Proteomics, School of Biotechnology, KTH-Royal Institute of Technology, Stockholm, Sweden

Science for Life Laboratory, KTH-Royal Institute of Technology Stockholm, Sweden

Department of Immunology, Genetics, and Pathology, Uppsala University, Uppsala, Sweden

Neuroimmunology Unit, Department of Clinical Neuroscience, Karolinska Institute, Stockholm, Sweden

Department of Molecular Toxicology, Safety Assessment, AstraZeneca R&D Södertälje, Sweden

Rheumatology Division, ProteoRed/ISCIII Proteomics Group, INIBIC – Hospital Universitario de A Coruña, Coruña, Spain

Protagen AG, Dortmund, Germany

REFERENCES

1. Kingsmore SF. Multiplexed protein measurement: technologies and applications of protein and antibody arrays. Nat Rev Drug Discov 2006 Apr;5(4):310-20.
2. Ellington AA, Kullo IJ, Bailey KR, Klee GG. Antibody-based protein multiplex platforms: technical and operational challenges. Clin Chem 2010 Feb;56(2):186-93.
3. Schwenk JM, Igel U, Neiman M, Langen H, Becker C, Bjartell A, Ponten F, Wiklund F, Grönberg H, Nilsson P, Uhlen M. Toward next generation plasma profiling via heat-induced epitope retrieval and array-based assays. Mol Cell Proteomics 2010 Nov;9(11):2497-507.
4. Bystrom S, Ayoglu B, Haggmark A, Mitsios N, Hong M-G, Drobin K, Forsstrom B, Fredolini C, Khademi M, Amor S, Uhlen M, Olsson T, Mulder J, Nilsson P, Schwenk JM. Affinity proteomic profiling of plasma, cerebrospinal fluid, and brain tissue within multiple sclerosis. Journal of Proteome Research 2014;13(11):4607-4619.
5. Schwenk J, Gry M, Rimini R, Uhlen M, Nilsson P. Antibody suspension bead arrays within serum proteomics. Journal Of Proteome Research 2008;7(8):3168-3179.
6. Schwenk J, Igel U, Kato B, Nicholson G, Karpe F, Uhlen M, Nilsson P. Comparative protein profiling of serum and plasma using an antibody suspension bead array approach. Proteomics 2010;10(3):532-540.
7. Rimini R, Schwenk J, Sundberg M, Sjoeberg R, Klevebring D, Gry M, Uhlen M, Nilsson P. Validation of serum protein profiles by a dual antibody array approach. Journal Of Proteomics 2009;73(2):252-266.
8. Kato BS, Nicholson G, Neiman M, Rantalainen M, Holmes CC, Barrett A, Uhlen M, Nilsson P, Spector TD, Schwenk JM. Variance decomposition of protein profiles from antibody arrays using a longitudinal twin model. Proteome Science 2011;9(U1-U16).
9. Ayoglu B, Haggmark A, Khademi M, Olsson T, Uhlen M, Schwenk JM, Nilsson P. Autoantibody profiling in multiple sclerosis using arrays of human protein fragments. Molecular & Cellular Proteomics 2013;12(9):2657–2672.
10. Haggmark A, Bystrom S, Ayoglu B, Qundos U, Uhlen M, Khademi M, Olsson T, Schwenk J M, Nilsson P. Antibody-based profiling of cerebrospinal fluid within multiple sclerosis. Proteomics 2013;13(15):2256–2267.
11. Qundos U, Johannesson H, Fredolini C, O'Hurley G, Branca R, Uhlen M, Wiklund F, Bjartell A, Nilsson P, Schwenk JM. Analysis of plasma from prostate cancer patients links decreased carnosine dipeptidase 1 levels to lymph node metastasis. Translational Proteomics 2014;2:14–24.
12. Hjelm B, Forsstroem B, Igel U, Johannesson H, Stadler C, Lundberg E, Ponten F, Sjoeberg A, Rockberg J, Schwenk JM, Nilsson P, Johansson C, Uhlen M. Generation of monospecific antibodies based on affinity capture of polyclonal antibodies. Protein Science 2011;20(11):1824-1835.
13. Darmanis S, Cui T, Drobin K, Li S-C, Oberg K, Nilsson P, Schwenk JM, Giandomenico V. Identification of candidate serum proteins for classifying well-differentiated small intestinal neuroendocrine tumors. PLoS One 2013;8(11):U1282-U1290.
14. Neiman M, Hedberg JJ, Doennes PR, Schuppe-Koistinen I, Hanschke S, Schindler R, Uhlen M, Schwenk JM, Nilsson P. Plasma profiling reveals human fibulin-1 as candidate marker for renal impairment. Journal Of Proteome Research 2011;10(11):4925-4934.
15. Ayoglu B, Chaouch A, Lochmueller H, Politano L, Bertini E, Spitali P, Hiller M, Niks EH, Gualandi F, Ponten F, Bushby K, Aartsma-Rus A, Schwartz E, Le Priol Y, Straub V, Uhlen M, Cirak S, Hoen PAC, Muntoni F, Ferlini A, Schwenk JM, Nilsson P, Szigyarto CA-K. Affinity proteomics within rare diseases: A bio-nmd study for blood biomarkers of muscular dystrophies. EMBO Molecular Medicine 2014;6(7):918–936.
16. Henjes F, Lourido L, Ruiz-Romero C, Fernandez-Tajes J, Schwenk JM, Gonzalez-Gonzalez M, Banco FJ, Nilsson P, Fuentes M. Analysis of autoantibody profiles in osteoarthritis using comprehensive protein array concepts. Journal of Proteome Research 2014;13(11):5218–5229.
17. Ayoglu B, Szarka E, Huber K, Orosz A, Babos F, Magyar A, Hudecz F, Rojkovich B, Gati T, Nagy G, Schwenk JM, Sarmay G, Prechl J, Nilsson P, Papp K. Bead arrays for antibody and complement profiling reveal joint contribution of antibody isotypes to c3 deposition. PLoS One 2014;9(5):U780-U792.

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