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Further Development of IgY-Immunoaffinity Fractionation – SuperMix and SepproTip Technologies

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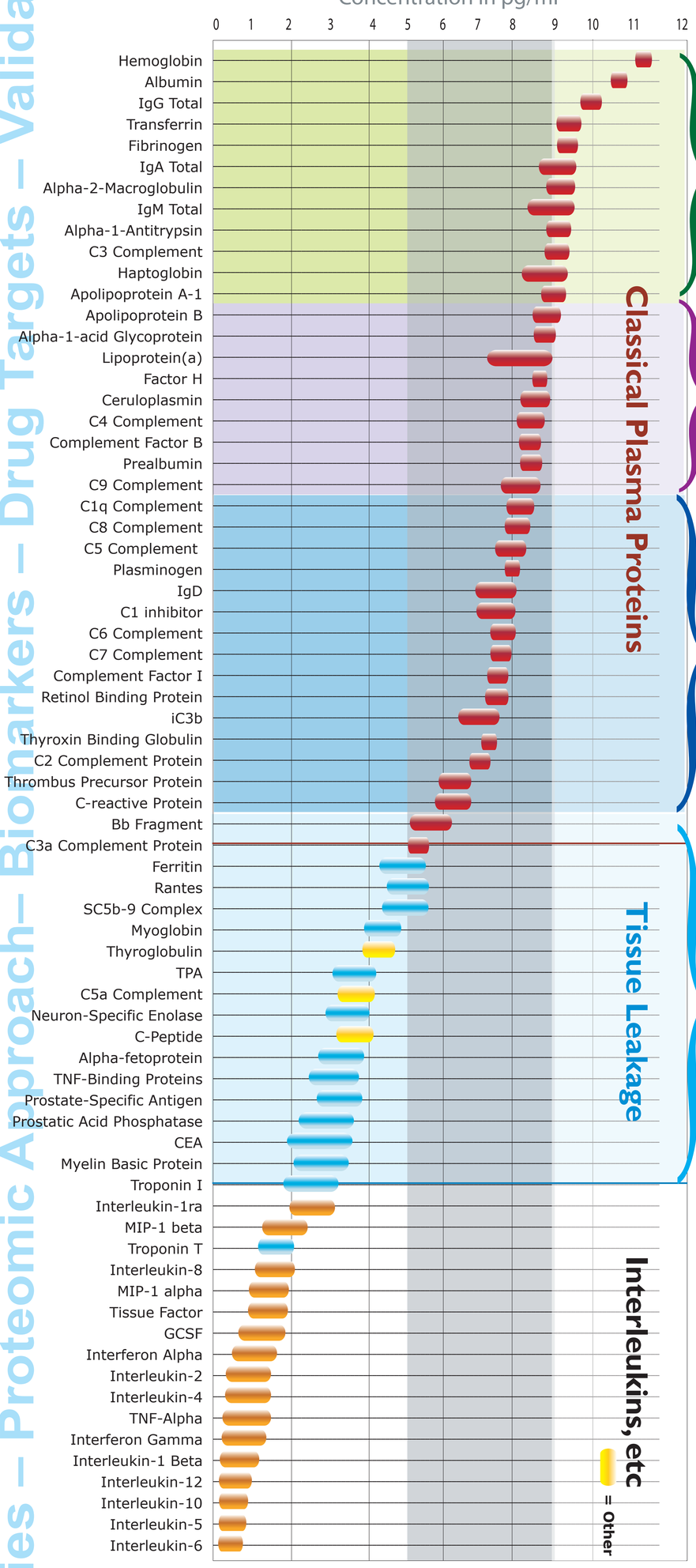
ABSTRACT

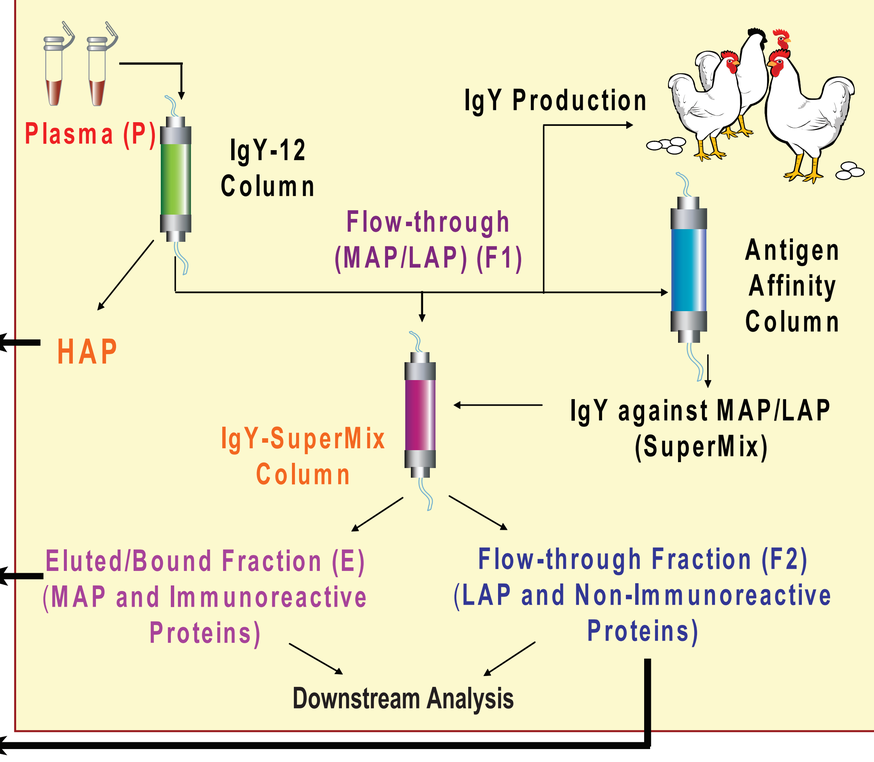
Avian polyclonal IgY (Immunoglobulin Yolk) antibodies have unique and advantageous features that allow for highly-specific and effective capture of protein targets. Previously, we have reported development and application of IgY microbeads for the one-step removal of highly-abundant proteins (HAP) from plasma using immunoaffinity columns. However, after removal of top HAP, the next level of moderately abundance proteins (MAP) becomes an obstacle to access low abundance proteins (LAP), where the majority of biologically interesting and clinically important biomarkers reside. To tackle this challenge, we further developed the IgY-microbead system by two approaches: (1) establishing a Plasma-SuperMix column system to separate MAP from LAP; (2) developing a Cell-SuperMix column system to enrich proteins from tissue leakage. These novel approaches enable deeper and more effective access into the population of LAP. By coupling HAP removal with SuperMix system, proteins less than 1ng/ml in plasma were detected by LC/MS/MS analysis. In addition to digging deeper, the SepproTip platform for automated, multiplex, and HTP sample preparation was developed. This system allows for processing 15 µl of each of 12 samples at a time with minimal hands-on manipulation. The turnaround time of 12 samples per 60 minutes allows large number of samples being processed without decrease in sample preparation quality. The SepproTip system makes "digging faster" possible for meeting the needs of HTP sample preparation.

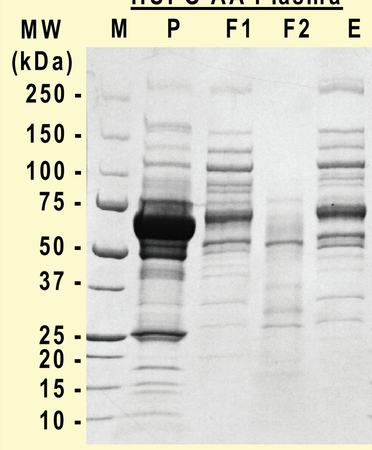
Proteins – Antibodies – Proteomic Approach– Biomarkers – Drug Targets – Validation

Dynamic Range of Human Plasma



Plasma Protein Profiling- Selective Removal of HAP and MAP

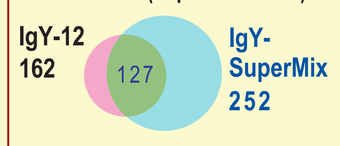




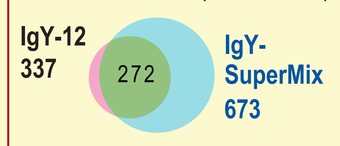
M: MW Marker
P: Plasma
F1: Flow-Through of IgY- 12
F2: Flow-Through of IgY- 12 + IgY-SuperMix
E: Eluate from SuperMix

4-20% SDS-PAGE under reducing conditions
8.4ug Protein loaded per lane

LC-MS/MS (triplicate runs)



SCX-LC-MS/MS (25 fractions)



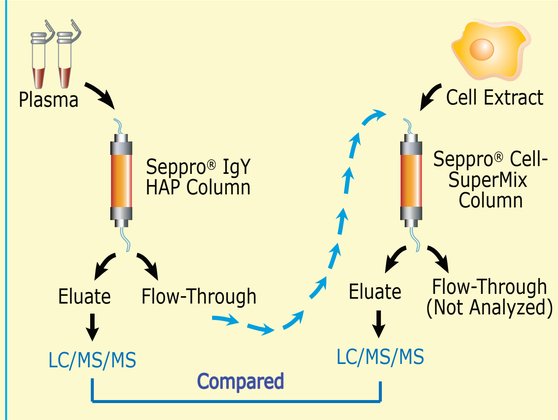
* ≥2 peptides per protein

Examples of LAP Identified
M-CSF (214pg/ml), ANGPT2, IGF1R, MBP, MMP8 (11ng/ml).

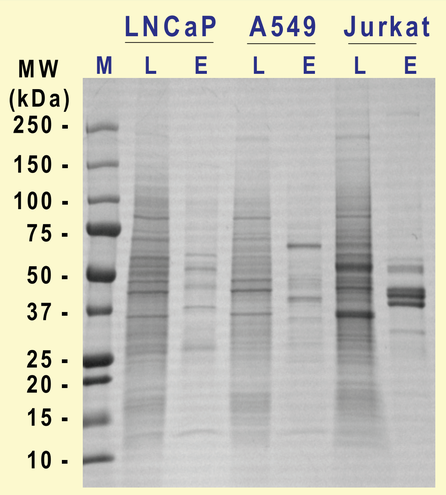
Note: About 2-fold increase of protein identification by IgY-12 and Plasma-SuperMix combination

Plasma Protein Profiling- Selective Enrichment of Tissue leakage

To characterize Cell-SuperMix column, each cell lysate was fractionated by corresponding Cell-SuperMix column. The eluate was further analyzed by 1DE and LC/MS/MS (LNCaP only). The next step is to test whether Cell-SuperMix column can enrich proteins of tissue leakage. Normal plasma sample was processed through LNCaP SuperMix column coupled with a IgY-14 column. Proteins bound to the SuperMix column was eluted and further analyzed by LC/MS/MS. The samples were first concentrated with Amicon ultra-15 concentrators (15 mL/5K cutoff, Millipore) followed by buffer exchange to 50 mM NH4HCO3. Protein concentration was then determined by Coomassie assay (Pierce). Proteins were then denatured with 50% tri-fluoroethanol (TFE) by incubation at 60oC for 2h, and reduced by 2 mM DTT for 1h at 37o C. Following a 5-fold dilution with 25mM NH4HCO3, proteins were digested by trypsin in an enzyme to protein ratio of 1:50 for 5h at 37oC. After digestion, peptides were lyophilized to dryness and reconstituted in 25mM NH4HCO3. Approximating 2 µg of peptides for each sample were loaded for LC-MS/MS analysis on a LTQ ion trap instrument.



1DE of Cell Proteins



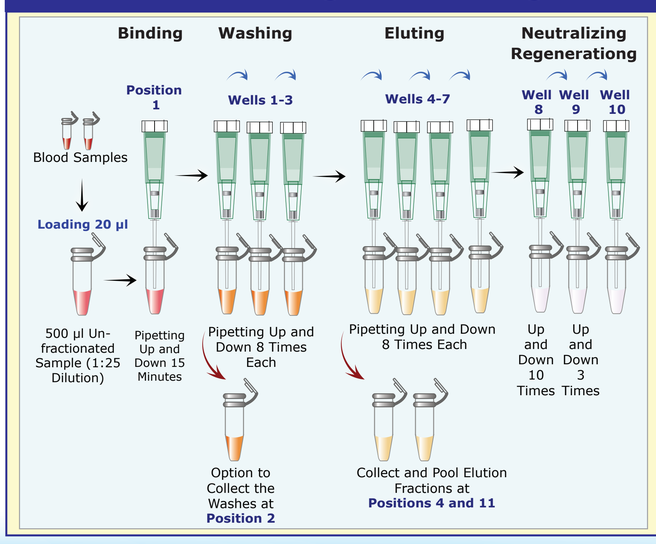
SDS PAGE Analysis of Cell Protein Extracts Processing through Cell-SuperMix Columns

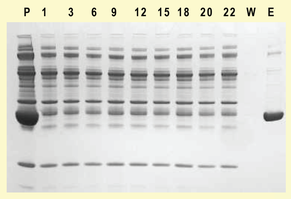
M: M W Marker
L: Totaly Cell Lysate (14-20µg proteins)
E: Eluate from Cell-SuperMix Column (2-3µg proteins

Proteins in both Cell & Plasma

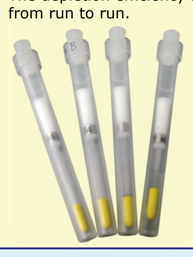
PROTEIN DESCRIPTION	SUBCELLULAR LOCATION
ISOFORM 1 OF PROTEIN FAM62A.	Cell membrane
ISOFORM PSMA-1 OF GLUTAMATE CARBOXYPEPTIDASE 2.	Cell membrane
NEPRILYSIN.	Cell membrane
ISOFORM A OF PROTEIN CUTA PRECURSOR.	Cell membrane
TUBULIN BETA-2C CHAIN.	Cytoplasma
KERATIN, TYPE I CYTOSKELETAL 10.	Cytoplasma
KERATIN, TYPE I CYTOSKELETAL 9.	Cytoplasma
PEROXIREDOXIN-2.	Cytoplasma
GLUCOSE-6-PHOSPHATE ISOMERASE.	Cytoplasma
TUBA6 PROTEIN.	Cytoplasma
ASPARTATE AMINOTRANSFERASE, CYTOPLASMIC.	Cytoplasma
KERATIN, TYPE II CYTOSKELETAL 1.	Cytoplasma
KERATIN, TYPE II CYTOSKELETAL 8.	Cytoplasma
49 KDA PROTEIN.	Cytoplasma
KERATIN, TYPE I CYTOSKELETAL 18.	Cytoplasma
STRESS-70 PROTEIN, MITOCHONDRIAL PRECURSOR.	Mitochondrial
DELTA3,5-DELTA2,4-DIENOYL-COA ISOMERASE, MITOCHONDRIAL PRECURSOR.	Mitochondrial
COMPLEMENT COMPONENT 1 Q SUBCOMPONENT-BINDING PROTEIN, MITOCHONDRIAL PRECURSOR.	Mitochondrial
ISOFORM MITOCHONDRIAL OF GLUTATHIONE REDUCTASE, MITOCHONDRIAL PRECURSOR.	Mitochondrial
PROHIBITIN.	Mitochondrial
DIHYDROLIPOYLLYSINE-RESIDUE ACETYLTRANSFERASE COMPONENT OF PYRUVATE DEHYDROGENASE COMPLEX, MITOCHONDRIAL PRECURSOR.	Mitochondrial
SHORT HEAT SHOCK PROTEIN 60 HSP60S2.	Mitochondrial
10 KDA HEAT SHOCK PROTEIN, MITOCHONDRIAL.	Mitochondrial
ISOFORM 1 OF 3,2-TRANS-ENOYL-COA ISOMERASE, MITOCHONDRIAL PRECURSOR.	Mitochondrial
ATP SYNTHASE SUBUNIT BETA, MITOCHONDRIAL PRECURSOR.	Mitochondrial
PYRROLINE-5-CARBOXYLATE REDUCTASE 1 ISOFORM 2.	Mitochondrial
ATP SYNTHASE SUBUNIT ALPHA, MITOCHONDRIAL PRECURSOR.	Mitochondrial
HEAT SHOCK PROTEIN 60.	Mitochondrial
18 KDA PROTEIN.	Nuclear membrane
C-MYC-RESPONSIVE PROTEIN RCL.	Nucleus
SPLICING FACTOR 3A SUBUNIT 2.	Nucleus
NCL PROTEIN.	Nucleus
ISOFORM 2 OF NUCLEOPHOSMIN.	Nucleus
PEROXISOMAL 3,2-TRANS-ENOYL-COA ISOMERASE.	Peroxisosome
CATALASE.	Peroxisosome
ISOFORM LONG OF BETA-GLUCURONIDASE PRECURSOR.	Lysosome
MACROPHAGE MIGRATION INHIBITORY FACTOR.	Secreted
GAMMA-GLUTAMYL HYDROLASE PRECURSOR.	Sectreted, Lysosome

Going Faster –High-Throughput – SepproTip





Human Plasma were processed using SepproTip IgY-HSA on APT12. The depletion efficiency is constant from run to run.



Conclusions

- Removal of Highly Abundant Protein (HAP) is an essential step to detect Low Abundant Proteins (LAP) in plasma by 2DE or MS. Immunoaffinity separation via IgY-microbead columns is the most effective tools for selective removal of HAP or selective enrichment of LAP.
- IgY Plasma-SuperMix was developed to partition the next level of Moderately Abundance Proteins (MAP). By coupling HAP removal with SuperMix column, this system enables identification of LAP and potential biomarkers at the level of less than 1ng/ml in plasma.
- IgY Cell-SuperMix system is a novel approach to specifically enrich LAP and to detect hidden biomarkers that are tissue leakage particularly under diseased conditions.
- SepproTip automation system enables high-throughput plasma sample preparation.