High-throughput, Automated INTip™ SPE Combined with AlbuSorb™ Products for Efficient Albumin and Albumin/IgG Depletion

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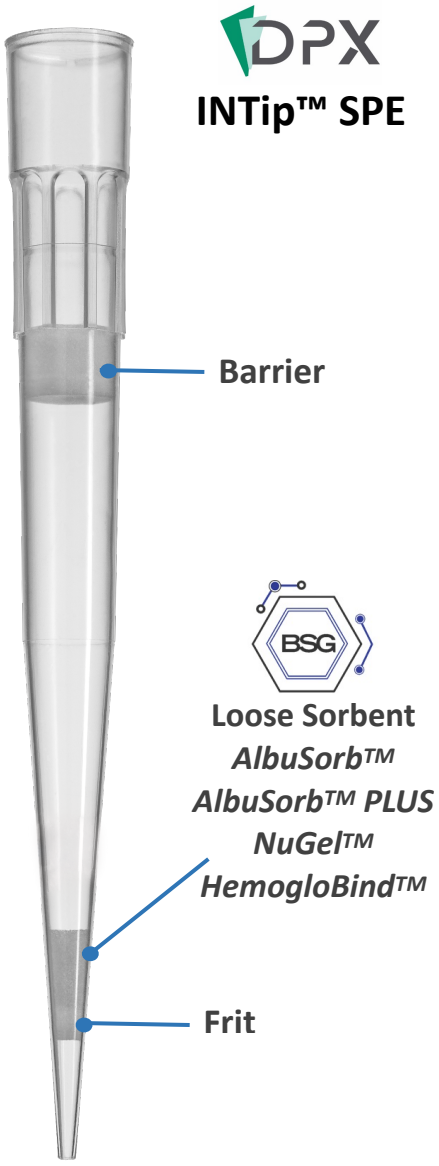
Introduction

As LC-MS proteomic analysis moves from discovery to the clinic, there is increasing need for high throughput automation in sample prep workflows. We report here new methods combining technologies to incorporate beads for depletion of highly abundant blood proteins, into pipette tips known as XTRaction tips or XTR tips. The XTR tip format improves ease of use and scalability to process multiple samples in parallel, utilizing 96-well plates and automated liquid handlers. The incorporated beads are consumable, and not derived from immuno-affinity. The beads are loosely contained inside the XTR tips for a dispersive functionality that maximizes depletion efficacy. Thus, this combination of technologies achieves fast, streamlined workflows that simply and robustly increase efficiencies necessary for clinical proteomic analysis of whole blood, serum and plasma.

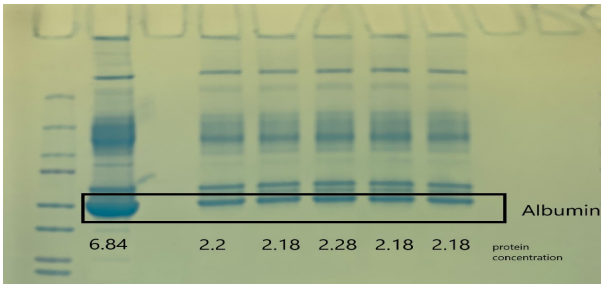
Methods

Biotech Support Group (BSG) supplies specialized beads for the selective depletion of Albumin (AlbuSorb™) and Albumin & IgG (AlbuSorb™ PLUS), and Hemoglobin (NuGel™ HemogloBind™), including all necessary buffers. DPX Technologies provides INTip(tm) SPE (XTR tips). This new method uses a serum volume of 20 µL and 65 mg of AlbuSorb ™ or AlbuSorb™ PLUS ™ for albumin or albumin/IgG depletion respectively. For Hemoglobin removal, 10 µL of whole blood lysate was used, and 40 mg of NuGel™ HemogloBind™ beads was incorporated into the XTR tips. Each bead type utilizes similar protocols, as described. The workflow was performed using an Integra VIAFLO with varied cycle times to establish optimal extraction performance.

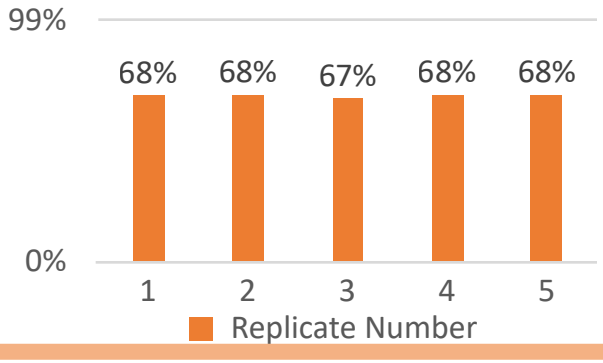


AlbuSorb™ Protocol & Results

5 replicates with 20 µL human serum and 50 mg AlbuSorb™ beads at 25 cycles of binding, using 300 µL Integra Tips, processed on the Integra Viaflo 96 platform. OD280 readings represent the approximate protein concentration.



Percentage Protein Reduction after AlbuSorb™



LC-MS Analysis Methods

In-gel digestion

Samples (~20 µg protein) were run by SDS-PAGE, bands excised and proteins reduced with 10 mM DTT for 30 min at 60℃, alkylated with 20 mM iodoacetamide for 45 min at room temperature in the dark and digested overnight with 0.5 µg of either trypsin (Pierce MS Grade, ThermoFisher) at 37℃. Peptides were extracted twice with 5% formic acid, 60% acetonitrile and dried under vacuum.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

LC-MS/MS was conducted using a nano LC (Dionex UltiMate™ 3000 RLSCnano System, ThermoFisher) interfaced with an Eclipse™ Tribrid™ mass spectrometer (ThermoFisher). Each sample (~25% of digests) was loaded onto a fused silica trap column (Acclaim PepMap 100, 75 µm x 2 cm, ThermoFisher). After washing for 5 min at 5 µl/min with 0.1% TFA, the trap column was brought in-line with an analytical column (nanoEase M/Z peptide BEH C18, 130Å, 1.7 µm, 75 µm x 250 mm, Waters) for LC-MS/MS. Peptides were fractionated at 300 nL/min using a segmented linear gradient of 4-15% B in 5 min (where A: 0.2% formic acid, and B: 0.16% formic acid, 80% acetonitrile), 15-25% B in 40 min, 25-50% B in 44 min, and 50-90% B in 11 min. Solution B then returns at 4% for 5 minutes for the next run. The scan sequence began with an MS1 spectrum (Orbitrap analysis, resolution 120,000, scan range from M/Z 375–1500, automatic gain control target 1×10^6, maximum injection time 100 ms). The top S (3 sec) duty cycle scheme was used to determine the number of MS/MS scans performed for each cycle. Precursor ions of charges 2-7 were selected for MS/MS and a dynamic exclusion of 60 sec was used to avoid repeat sampling. Precursor ions were isolated in the quadrupole with an isolation window of 1.2 m/z, automatic gain control target 1×10^5, and fragmented with higher-energy collisional dissociation with a normalized collision energy of 30%. Fragments were scanned in Orbitrap with resolution of 15,000. MS/MS scan ranges were determined by the charge state of the parent ion but a lower limit was set to 110 m/z.

Database Search

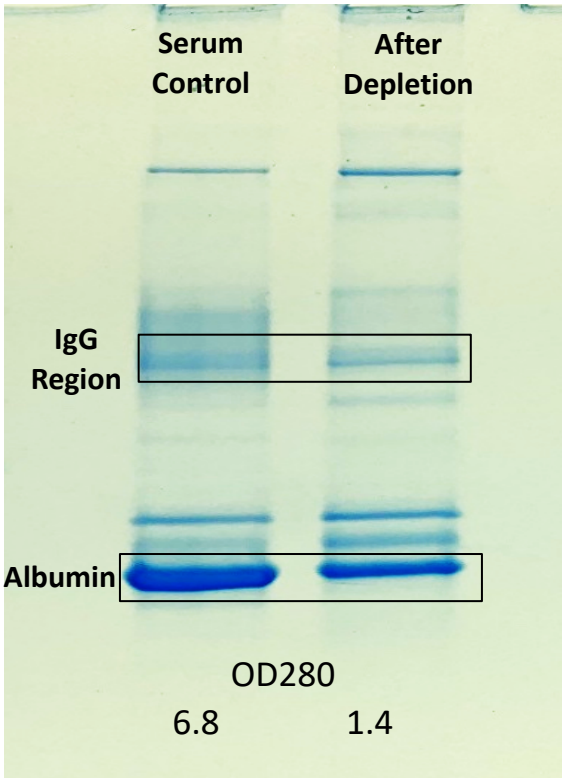
Peak list MASCOT Generic Format (MGF) files were generated by Thermo Proteome Discoverer (v. 2.1) and searched against *E.coli* database from NCBI, the Uniprot human reference proteome database and a database composed of common lab contaminants (CRAP) using an in-house installation of GPM Fury[1] (X!Tandem Alanine). Mudpit searches were conducted using all mgf files from LC-MS/MS analysis of digests from a given type of protease. Parameters for the initial search were as follows: parent mass error ± 7 ppm; fragment mass error ± 20 ppm; fixed modification of carbamidomethylation on cysteine and variable modifications of methionine monooxidation, serine, threonine and tyrosine phosphorylation. Variable modifications during three rounds of refinement besides serine, threonine and tyrosine phosphorylation for each round, were as follows: 1st round: monooxidation at methionine and tryptophan, 2nd round: deamination at glutamine and asparagine; 3rd round: dioxidation at methionine and tryptophan. Protease specificity was set to trypsin (C-terminal of R/K unless followed by P). There was 1 allowed missed cleavage during the preliminary search and 5 during refinement. Minimum acceptable peptide and protein expectation scores were set at 10^{-2} and 10^{-4}, respectively. The overall peptide false positive rate[2] was 0.07%.

[1]Craig R. & Beavis RC. TANDEM: matching proteins with tandem mass spectra. Bioinformatics. 2004 20:1466-7.
[2]Gupta N, et al., J Am Soc Mass Spectrom. 2011 22:1111-20

AlbuSorb™ PLUS Protocol & Results

65 mg AlbuSorb™ PLUS in Integra 300 µL tip

1. 5cycles of 250 µL pipetting in 300 µL binding buffer to wet sorbent
2. 5 cycles of 250 µL pipetting in 300 µL fresh binding buffer
3. 3. 20 µL human serum diluted in 200 µL binding buffer for total sample volume of 220 µL to create sample for depletion
4. 25 cycles of 200 µL pipetting for albumin and IgG removal
5. Retain depleted sample for analysis



Protein Category	Approx Serum Concentration %	AlbuSorb™PLUS/INTip LC-MS Spectral Count Analysis (% of Total)	Remarks
Albumin	50	16%	
Immunoglobulins	20		Categorizing Ig variable regions is challenging
IgG		10%	AlbuSorb™ PLUS uses an optimized Protein A, much more specific for IgGs than other immunoglobulins
Combined IgM, IgA, IgD		3%	
Apolipoproteins	4	10%	
Complement & Coagulation related	10		
C3	3	9%	
Other Complement-related & Coagulation/Fibrinolysis	7	4%	BSG has observed in prior work that Complement (except C3) and Coagulation proteins interact on beads & tend to deplete as a group
Other Proteins	6	48%	3X enrichment relative to Albumin
# of Unique Protein IDs (≥2 Sp. Cts)		220	

NuGel™ HemogloBind ™ Protocol & Results

1. Dilute 10 µL whole blood in 200 µL HB buffer to create hemolyzed sample for depletion

2. Let stand 10 minutes to prepare sample for binding / removal of hemoglobin

3. 40 mg NuGel™ HemogloBind™ matrix in Integra 300 µL tip

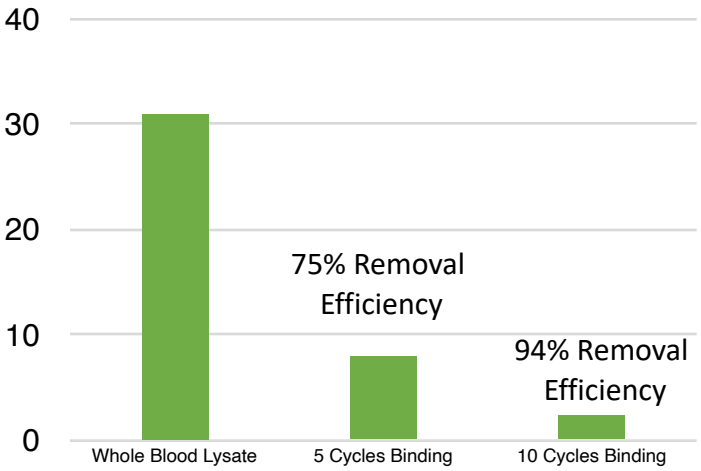
4. 2-300 µL aliquots of HB buffer

5. Pipette 250 µL for 5 cycles in aliquot 1

6. Repeat 5 cycles of 250 µL volume in aliquot 2

7. Pipette 200 µL in sample for 5 to 10 cycles of binding to remove hemoglobin

Hemoglobin Depletion OD414 (Avg 2 readings)



	Whole Blood Lysate Control	After NuGel™ HemogloBind™/ INTip™
Total Spectral Counts (Sp. Cts)	14532	19054
Total Protein IDs (≥2 Sp. Cts)	306	420
# of Unique Protein IDs (≥2 Sp. Cts)	27	134
# of Enriched Proteins ≥3X		47
# of Depleted Proteins (other than Hemoglobin subunits) ≥3X	6	

Representative Enriched Proteins after NuGel HemogloBind (Uniprot ID)	Description	Sp. Cts control	Sp. Cts after
sp|P01023|A2MG_HUMAN	Alpha-2-macroglobulin	376	896
sp|P04114|APOB_HUMAN	Apolipoprotein B-100	280	660
sp|P02751-1|FINC_HUMAN	Fibronectin	120	248
sp|P43652|AFAM_HUMAN	Afamin	23	54
sp|P11277|SPTB1_HUMAN	Spectrin beta chain	32	130
sp|P02549|SPTA1_HUMAN	Spectrin alpha chain	39	163
sp|P04003|C4BPA_HUMAN	C4b-binding protein alpha chain	15	50
sp|P08697|A2AP_HUMAN	Alpha-2-antiplasmin	19	48
sp|Q06033|ITIH3_HUMAN	Inter-alpha-trypsin inhibitor heavy chain	14	43
sp|P02730|B3AT_HUMAN	Band 3 anion transport protein	42	110
sp|P05543|THBG_HUMAN	Thyroxine-binding globulin	7	27
sp|P09871|C1S_HUMAN	Complement C1s subcomponent	9	34
sp|O75882|ATRN_HUMAN	Attractin	4	18
sp|P16157|ANK1_HUMAN	Ankyrin-1	18	73
sp|P04406|G3P_HUMAN	Glyceraldehyde-3-phosphate	28	61
sp|P02750|A2GL_HUMAN	Leucine-rich alpha-2-glycoprotein	4	13
sp|P62258|1433E_HUMAN	14-3-3 protein epsilon	11	51
sp|P20742|PZP_HUMAN	Pregnancy zone protein	7	36
tr|G3V1D3|G3V1D3_HUMAN	Dipeptidyl peptidase 3	9	42
sp|P80108|PHLD_HUMAN	Phosphatidylinositol-glycan-specific	1	10
tr|C9JIF9|C9JIF9_HUMAN	Acyl-peptide hydrolase	11	38
sp|P15169|CBPN_HUMAN	Carboxypeptidase N catalytic chain	2	14
sp|P05160|F13B_HUMAN	Coagulation factor XIII B chain	1	13
sp|P16452|EPB42_HUMAN	Protein 4.2	5	38
sp|Q13630|FCL_HUMAN	GDP-L-fucose synthase	8	33
sp|P09960|LKHA4_HUMAN	Leukotriene A-4 hydrolase	9	28
sp|P22314-2|UBA1_HUMAN	Ubiquitin-like modifier-activating enzyme	8	28
sp|P07900|HS90A_HUMAN	Heat shock protein HSP 90-alpha	3	28
sp|P12955|PEPD_HUMAN	Xaa-Pro dipeptidase	3	23
sp|P23142|FBLN1_HUMAN	Fibulin-1	4	19
sp|P25786|PSA1_HUMAN	Proteasome subunit alpha type-1	2	25
sp|P31946|1433B_HUMAN	14-3-3 protein beta/alpha	4	18
sp|P45974|UBP5_HUMAN	Ubiquitin carboxyl-terminal hydrolase 5	2	20
sp|Q6XQN6|PNCB_HUMAN	Nicotinate phosphoribosyltransferase	3	17
sp|P09211|GSTP1_HUMAN	Glutathione S-transferase P	5	15
sp|P61970|NTF2_HUMAN	Nuclear transport factor 2	4	15
tr|F6TLX2|F6TLX2_HUMAN	Glyoxalase domain-containing protein 4	4	15
sp|P07996|TSP1_HUMAN	Thrombospondin-1	nd	19
sp|P28070|PSB4_HUMAN	Proteasome subunit beta type-4	4	15
sp|P00488|F13A_HUMAN	Coagulation factor XIII A chain	1	16
sp|P21980|TGM2_HUMAN	Protein-glutamine gamma-	1	14
sp|Q14974|IMB1_HUMAN	Importin subunit beta-1	nd	14
sp|P53396|ACLY_HUMAN	ATP-citrate synthase	1	13
sp|P06132|DCUP_HUMAN	Uroporphyrinogen decarboxylase	1	13
tr|B3KQV6|B3KQV6_HUMAN	Serine/threonine-protein phosphatase	nd	10

Conclusions

The optimal workflow consists of between 10 to 25 aspirate/dispense steps with total processing time of approximately 20-30 minutes. These methods have minimal hands-on requirements. The final depleted sample volume recovery was approximately 200 µL. We demonstrate comparable depletion efficiencies to those pre-established by BSG for microfuge spin-filter formats. For Albumin and IgG, depletion was consistently within a 70-80% range, and sub-proteome LC-MS analysis revealed between 220-400 protein identifications, and sub-proteome enrichments of 3-5X. For AlbuSorb™, BSA was monitored at 50 mg/mL and was shown to be depleted to >90%; total protein depletion from human serum was shown to be >80%. For AlbuSorb™ PLUS, Albumin and IgG depletion percentage in human serum is estimated to be ~70%. For NuGel™ HemogloBind™, free Hemoglobin depletion is estimated to be >90%. Furthermore, we demonstrate exquisite reproducibility of depletion of Albumin and IgG in five human serum replicates.

The INTip™ SPE formats can be applied to the full range of BSG products, including Albumin & IgG removal, and Hemoglobin removal. As they do not rely on immuno-affinity, they are consumable and cost-effective. Optimization for specific applications can include bead amounts, sample volume loads, and cycle times. Functional integrity of all proteins is maintained, and downstream proteomic analysis can include LC-MS, immunoassays, cellular response and enzymatic assays.

Finally, INTip™ SPE formats have been proven to be compatible with most automation platforms, i.e., Integra, Hamilton, etc. As blood-derived samples for clinical proteomics progresses, simple, robust and reproducible sample preparation for targeted analysis will require consumable formats and compatibility with automation. The products and methods described here are uniquely fit for that purpose.

For more information, see our joint press release: https://www.biotechsupportgroup.com/Articles.asp?ID=615