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A Single Injection LC-MS Analysis Scheme for Simultaneous Analysis of Biotherapeutics and Host-Cell Impurities via Online Digestion LC-MS/MS

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Novel Aspect

Fully automated online protein digestion, affinity pulldown, intact mass analysis, peptide mapping of target therapeutics and host-cell proteins in a single analysis

Introduction

Analysis of protein drug targets and impurities via LCMS involves time consuming offline protein digestion and separation. Automated protein digestion systems have addressed concerns of irreproducibility and improved laboratory efficiency by reducing human interaction with the sample. Although peptide mapping and Immuno-MS workflows are well established for on-line digestion, more complicated, orthogonal analyses have yet to be explored. Protein digestion of analyte proteins can be performed with or without affinity pulldown (depletion) of target proteins to enhance recovery of impurity proteins. A configuration change allows separation and intact mass data of proteins to be acquired in addition to peptide data from the same sample by employing two parallel flow paths

Methods

Perfinity Workstation

LCMS 9030 QTOF

Figure 1. Sample preparation and methods

- Human IGG (Sigma-Aldrich, St. Louis, MO)
- 293 HEK Cell Lysate (Janssen R&D, Spring House, PA)
- Mobile Phases A/B: 0.1% Formic in Water, 0.1% Formic in Acetonitrile, (Sigma-Aldrich, St. Louis, MO)
- RP Column (peptide separation): 2.1x50mm C18 Aeris peptide Widepore (Phenomenex, CA)
- RP Column (protein separation): Restek Ultra C4 column (5µm 150x2.1mm)
- Affinity capture column: Protein A column (Perfinity BioSciences, West Lafayette, IN)
- Gradient: 2% to 35% Acetonitrile at 0.15 mL/min over 90 minutes for QTOF experiments
- MS system: Shimadzu 9030 QTOF (Shimadzu, Kyoto, Japan)
- LC system: Perfinity Workstation (Shimadzu, Kyoto, Japan and Perfinity BioSciences, West Lafayette, IN)
- Proprietary Perfinity Buffers for Digestion, Affinity Loading, Affinity Elution enable online protein digestion, affinity capture and depletion for analysis of human IGG and HEK 293 cell lysate mixtures.
- Digestion conditions were four minutes at 50 degrees centigrade.

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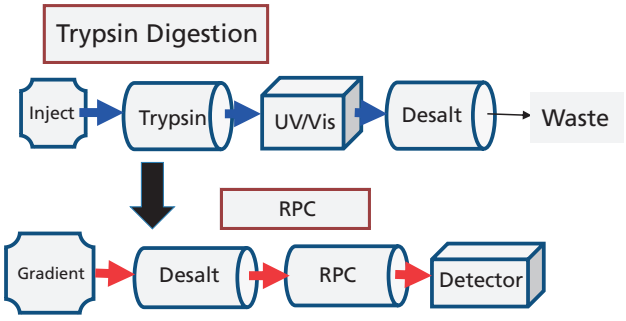
Results and Discussion

Experimental Set-up | Total Ion Chromatograms

A Trypsin Digestion and LCMS



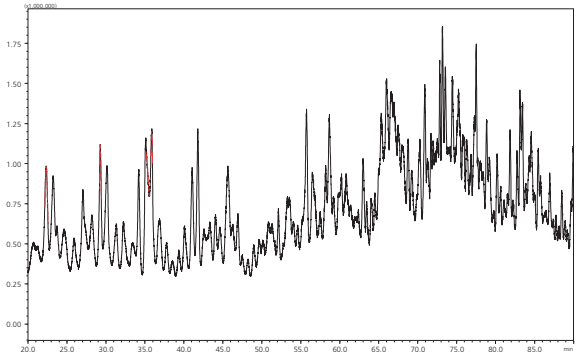
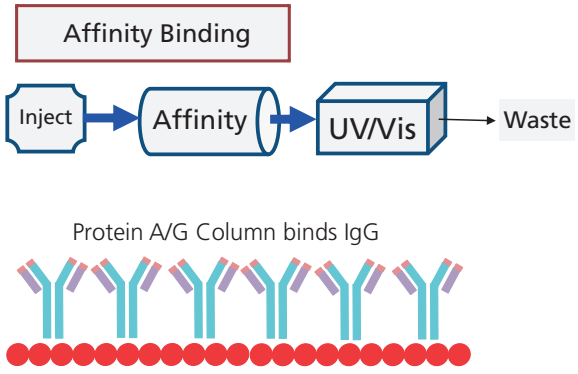


Figure 3. TIC: Peptides from online digestion of Undiluted HEK293 Cell Lysate only

B Affinity Capture/ HCP elution



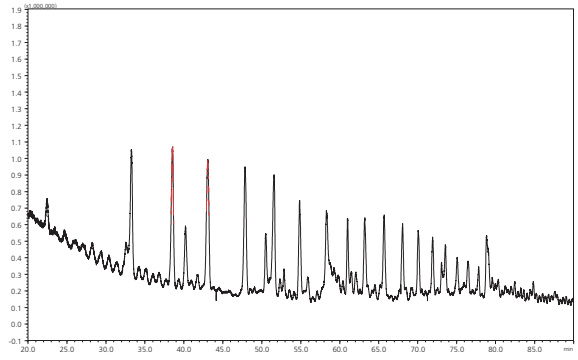


Figure 4. TIC: Peptide from depletion of IgG, followed by online digestion of HEK293 cell lysate. No IgG peptides were detected by MS

C Affinity Elution of IgG

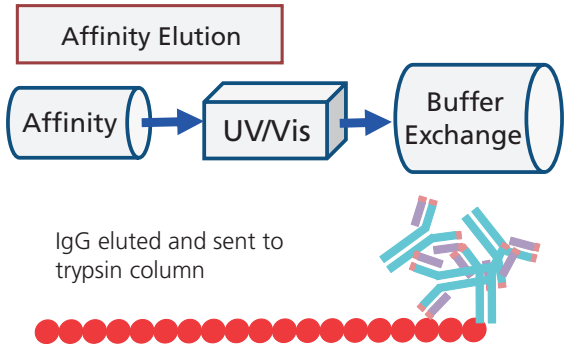


Figure 2. Pictorial representations for multiplexed experiments of mAbs and host cell impurities

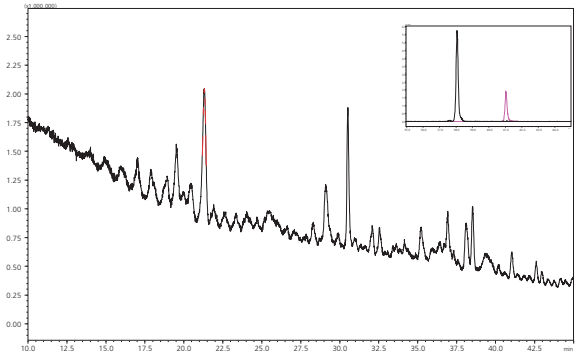


Figure 5. TIC: Peptides from affinity capture on protein A/G and elution of IgG with HEK293 cell lysate sent to waste. Inset is XIC of two IgG peptides

Cell Lysate Proteins Identified

Online-Digestion

Protein	XIC area summed
NU205_ Nuclear pore complex protein Nup205	1.57E+05
ACTBL_ Beta-actin-like protein 2	8.09E+04
HNRPD_ Heterogeneous nuclear ribonucleoprotein D0	5.74E+04
DX39B_ Spliceome RNA helicase DDX39B	4.79E+04
PRDX1_ Peroxiredoxin-1	5.46E+04
FKBP4_ Peptidyl-prolyl cis-trans isomerase FKBP4	4.84E+04
EF1A1_ Elongation factor 1-alpha 1	1.12E+06
IF5A1_ Eukaryotic translation initiation factor 5A-1	5.29E+04
PPIA_ Peptidyl-prolyl cis-trans isomerase A	2.48E+05
RS28_ 40S ribomal protein S28	1.22E+05
RAN_ GTP-binding nuclear protein Ran	1.06E+05
1433E_ 14-3-3 protein epsilon	7.53E+04
CH10_ 10 kDa heat shock protein, mitochondrial	6.52E+04
ARF3_ ADP-ribylation factor 3	5.17E+04
TPIS_ Triephphate isomerase	1.05E+05
MTPN_ Myotrophin	6.73E+04
SUMO3_ Small ubiquitin-related modifier 3	1.39E+05
KAD2_ Adenylate kinase 2, mitochondrial	1.86E+05
RAB28_ Ras-related protein Rab-28	1.06E+05
F10A1_ Hsc70-interacting protein	1.01E+05
NEST_ Nestin	1.40E+06
RANG_ Ran-specific GTPase-activating protein	5.77E+04
AN32A_ Acidic leucine-rich nuclear phphoprotein 32 family member A	1.01E+05
GRP75_ Stress-70 protein, mitochondrial	2.73E+05
HS71L_ Heat shock 70 kDa protein 1-like	8.67E+04
PRDX2_ Peroxiredoxin-2	5.08E+04
STIP1_ Stress-induced-phphoprotein 1	3.76E+04
PDIA3_ Protein disulfide-isomerase A3	5.13E+04
CALX_ Calnexin	8.00E+04
CALR_ Calreticulin	5.17E+04
EF1B_ Elongation factor 1-beta	6.47E+04
ROA2_ Heterogeneous nuclear ribonucleoproteins A2/B1	9.15E+04
NDKB_ Nucleide diphphate kinase B	5.22E+04
UBA1_ Ubiquitin-like modifier-activating enzyme 1	3.97E+04
PTMS_ Parathymin	4.76E+04
NUCL_ Nucleolin	1.29E+05
PGAM1_ Phphoglycerate mutase 1	9.58E+04
STMN1_ Stathmin	7.68E+04
KPYM_ Pyruvate kinase PKM	5.24E+04

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Online-Digestion

Protein	XIC area summed
KCRB_ Creatine kinase B-type	2.35E+05
IMDH2_ Inine-5'-monophphate dehydrogenase 2	4.16E+04
HSP7C_ Heat shock cognate 71 kDa protein	1.14E+05
GRP78_ 78 kDa gluce-regulated protein	4.48E+04
CH60_ 60 kDa heat shock protein, mitochondrial	6.77E+04
THIO_ Thioredoxin	1.16E+05
HS71B_ Heat shock 70 kDa protein 1B	1.07E+05
GSTP1_ Glutathione S-transferase P	3.78E+04
ENOG_ Gamma-enolase	6.85E+05
RSSA_ 40S ribomal protein SA	5.11E+04
ANXA5_ Annexin A5	5.78E+04
PROF1_ Profilin-1	1.14E+05
ACBP_ Acyl-CoA-binding protein	5.11E+04
NPM_ Nucleophmin	7.51E+04
ENOA_ Alpha-enolase	7.75E+04
KCRM_ Creatine kinase M-type	9.07E+04
LA_ Lupus La protein	5.76E+04
RLA2_ 60S acidic ribomal protein P2	6.74E+04
G3P_ Glyceraldehyde-3-phphate dehydrogenase	1.56E+05
VWF_ von Willebrand factor	8.61E+04
ALDOA_ Fructe-bisphphate aldolase A	1.40E+05
RPP40_ Ribonuclease P protein subunit p40	6.17E+04
BAF_ Barrier-to-autointegration factor	6.29E+04
PDCD5_ Programmed cell death protein 5	6.39E+04

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Affinity Capture /HCP Elution

Protein	XIC area summed
RHOF_ Rho-related GTP-binding protein RhoF	1.15E+05
IF5A2_ Eukaryotic translation initiation factor 5A-2	8.97E+04
ARHG5_ Rho guanine nucleotide exchange factor 5	5.84E+05
EF1A1_ Elongation factor 1-alpha 1	8.73E+06
ACTG_ Actin, cytoplasmic 2	3.39E+04
PPIA_ Peptidyl-prolyl cis-trans isomerase A	6.66E+04
RAN_ GTP-binding nuclear protein Ran	5.87E+04
H4_ Histone H4	8.99E+04
1433E_ 14-3-3 protein epsilon	1.34E+05
ARF3_ ADP-ribylation factor 3	7.67E+04
HSP72_ Heat shock-related 70 kDa protein 2	8.12E+04
NEST_ Nestin	1.66E+06
GRP75_ Stress-70 protein, mitochondrial	4.11E+04
TAGL2_ Transgelin-2	5.91E+04
HS71L_ Heat shock 70 kDa protein 1-like	4.11E+04
H2B1B_ Histone H2B type 1-B	6.34E+04
PRDX2_ Peroxiredoxin-2	8.67E+04
PDIA3_ Protein disulfide-isomerase A3	3.79E+04
UBA1_ Ubiquitin-like modifier-activating enzyme 1	5.55E+04
STMN1_ Stathmin	5.45E+04
MIF_ Macrophage migration inhibitory factor	2.16E+05
KCRB_ Creatine kinase B-type	5.08E+04
CH60_ 60 kDa heat shock protein, mitochondrial	6.44E+04
HS71B_ Heat shock 70 kDa protein 1B	5.72E+04
H2AZ_ Histone H2A.Z	3.89E+05
GSTP1_ Glutathione S-transferase P	1.14E+05
ADRB1_ Beta-1 adrenergic receptor	8.52E+04
PROF1_ Profilin-1	9.28E+04
NPM_ Nucleophmin	6.81E+04
ENOA_ Alpha-enolase	3.71E+04
LA_ Lupus La protein	3.92E+04
RLA2_ 60S acidic ribomal protein P2	7.49E+04
RLA1_ 60S acidic ribomal protein P1	2.00E+05
G3P_ Glyceraldehyde-3-phphate dehydrogenase	7.45E+04
IGHG1_ Immunoglobulin heavy constant gamma 1	7.02E+04

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Affinity Elution of IgG

Protein	Sequence	XIC area summed
IgG LC Sigma	K.DSTYSLSSTLTLSK.A	1.01E+05
IgG LC Sigma	K.VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK.A	1.52E+05
IgG LC Sigma	R.TVAAPSVFIFPPSDEQLK.S	4.48E+05
IgG HC Sigma	K.GFYPSDIAVEWESNGQPEnNYK.T	1.65E+05
IgG HC Sigma	K.GFYPSDIAVEWESNGQPEnNYK.T	7.16E+04
IgG HC Sigma	K.ALPAPIEK.T	1.00E+06
IgG HC Sigma	K.ALPAPIEK.T	1.96E+06
IgG HC Sigma	K.DTLMISR.T	3.44E+05
IgG HC Sigma	K.DTLMISR.T	5.49E+05
IgG HC Sigma	K.FNWYVDGVEVHNAK.T	1.89E+06
IgG HC Sigma	K.FNWYVDGVEVHNAK.T	1.37E+06
IgG HC Sigma	K.GFYPSDIAVEWESNGQPENNYK.T	6.20E+05
IgG HC Sigma	K.GFYPSDIAVEWESNGQPENNYK.T	2.80E+05
IgG HC Sigma	K.GPSVFPLAPSSK.S	8.72E+04
IgG HC Sigma	K.GPSVFPLAPSSK.S	4.48E+05
IgG HC Sigma	K.SLSLSPG.K	1.36E+06
IgG HC Sigma	K.TTPPVLDSDGSFFLYSK.L	5.95E+05
IgG HC Sigma	R.EPQVYTLPPSR.D	4.06E+05
IgG HC Sigma	R.TPEVTCVVVDVSHEDPEVK.F	5.69E+04
IgG HC Sigma	R.TPEVTCVVVDVSHEDPEVK.F	7.43E+04
IgG HC Sigma	R.VVSVLTVLHQDWLNGK.E	2.33E+05
IgG HC Sigma	R.VVSVLTVLHQDWLNGK.E	1.99E+05
IgG HC Sigma	R.VVSVLTVLHQDWLNGKEYK.C	9.09E+04
Immunoglobulin heavy variable 3-74	K.NTLYLQMNSLR.A	4.94E+04
Immunoglobulin lambda-like polypeptide 5	K.VTVLGQPK.A	1.72E+05
Immunoglobulin lambda-like polypeptide 5	K.ANPTVTLFPPSSEELQANK.A	1.11E+05
Immunoglobulin kappa variable 3-20	R.FSGSGSGTDFTLTISR.L	8.77E+04
Immunoglobulin heavy variable 3-13	K.NSLYLQMNSLR.A	2.94E+04
Immunoglobulin heavy constant gamma 2	K.GLPAPIEK.T	1.00E+06
Immunoglobulin heavy constant gamma 2	R.VVSVLTVVHQDWLNGKEYK.C	9.09E+04
Immunoglobulin heavy constant gamma 2	K.GLPAPIEK.T	5.40E+05
Immunoglobulin heavy constant gamma 2	K.GLPAPIEK.T	7.81E+05
Immunoglobulin heavy constant gamma 2	K.TTPPMLDSDGSFFLYSK.L	1.25E+06
Immunoglobulin heavy constant gamma 2	R.EPQVYTLPPSREEMTK.N	6.51E+05
Immunoglobulin heavy constant gamma 2	R.EPQVYTLPPSREEMTK.N	3.94E+05
Immunoglobulin heavy constant gamma 2	R.VVSVLTVVHQDWLNGK.E	3.37E+05
Immunoglobulin heavy constant gamma 2	R.VVSVLTVVHQDWLNGK.E	2.96E+05
Immunoglobulin kappa variable 3-11	R.LLIYDASNR.A	3.38E+04
Immunoglobulin kappa variable 2-30	R.FSGSGSGTDFTLK.I	3.99E+04
Trypsin-1	K.TLNnDIMLIK.L	1.89E+05
Trypsin-1	K.TLnNDIMLIK.L	1.89E+05

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Affinity Elution of IgG

Protein	Sequence	XIC area summed
Immunoglobulin lambda constant 2	K.AAPSVTLFPPSSEELQANK.A	2.81E+05
Immunoglobulin lambda constant 2	K.YAASSYLSLTPEQWK.S	1.51E+05
Nestin OS=Homo sapiens	K.EEGEEGEEECGR.D	2.40E+06
Immunoglobulin lambda variable 3-21	R.FSGSNSGNTATLTISR.V	2.94E+04
Peptidyl-prolyl cis-trans isomerase-like 2	R.VVGGFDVLTAMENVESDPK.T	5.93E+04
Nuclear pore complex protein Nup93	R.cDVTDNQSEVADK.T	1.08E+05
T-cell immunomodulatory protein	R.NDLIVFLADQNAPYFK.P	8.10E+04
Coiled-coil-helix-coiled-coil-helix domain-containing protein 1	K.PLILANR.V	6.21E+04
Pseudokinase FAM20A	R.LSVPNPWIRSYTIAGK.E	1.99E+05
Arf-GAP with GTPase, ANK repeat and PH domain-containing protein 3	R.ELRIETIAASSTPTPIR.K	3.86E+04
Anaphase-promoting complex subunit 2	R.LGLLmGTGAQGLR.E	1.40E+05

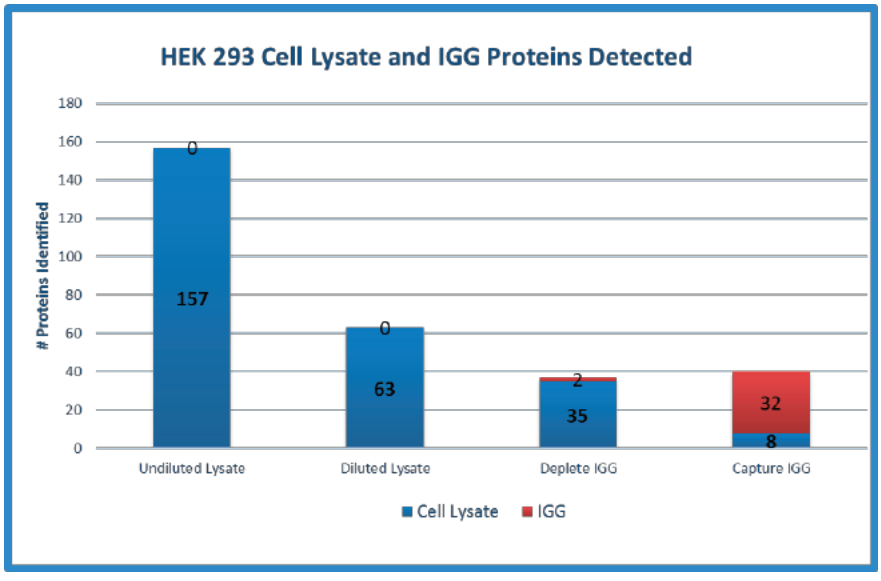


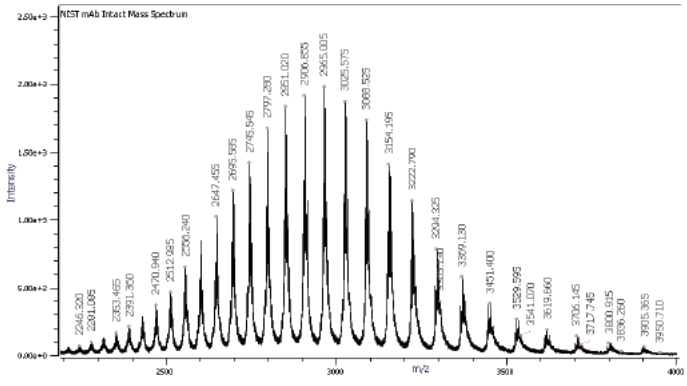
Figure 6. Number of cell lysate and IgG proteins identified in undiluted and diluted (four fold) cell lysate as well as in IgG/cell lysate mixture.

Experimental Observations

- Undiluted Cell Lysate was digested at a concentration of 20 µg of total protein Diluted Cell Lysate was a four fold dilution or 5 µg of total protein
- Human IgG was depleted from a sample containing 10 µg each of human IgG and HEK293 cell lysate proteins.
- A protein A/G column was used to deplete human IgG from solution allowing digestion of non-bound cell lysate proteins
- Affinity capture of IgG with cell lysate protein sent to waste demonstrates that IGG or a corresponding mAb can be selectively removed from host cell protein contaminates.
- Either digestion of IgG or intact mass analysis via LCMS can be performed online after the affinity capture step
- Low-levels of cell lysate proteins observed in the IgG affinity capture experiment could be hitch hiker proteins or unremoved proteins
- Further method modifications may be necessary to increase digestion of unbound proteins.
- Intact mass analysis of 1mg/mL solution of NIST mAb was performed using a Nexera LC system (not Perfinity) with the LCMS-9030 QTOF.

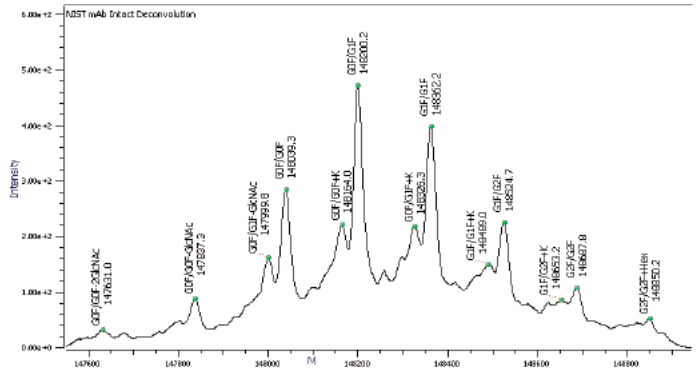
Results: Intact Mass Analysis of IgG

A



Mass Spectrum

B



Deconvoluted Spectrum

Figure 7 Intact Mass Analysis (A) Profile mode spectrum of NIST antibody on LCMS 9030 QTOF
(B) Deconvolution of NIST antibody Profile mode spectrum with Protein Metrics software

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Summary

- The Perfinity Workstation and a Shimadzu QTOF instrument performed online digestion and LCMS analysis of model host cell proteins. Digestion of two HEK293 cell lysate samples identified many high abundance proteins.
- Additionally, affinity capture or depletion of IgG from a mixture of HEK293 cell lysate and IgG was performed with the Perfinity Workstation, which allowed online digestion and analysis of either cell lysate proteins or IgG
- Affinity depletion of IgG effectively removed IgG from the sample allowing digestion and analysis of unbound cell lysate proteins.
- Affinity capture of IgG removed the majority of the cell lysate proteins so that digested peptides were predominantly from IgG.
- Intact mass analysis of the NIST mAb demonstrates using the LCMS-9030 demonstrates the possibility of collecting both intact mass and peptide mapping data from the same sample.

Future Directions

Development of additional protein digestion procedures and affinity capture/depletion for therapeutically relevant drugs of interest.

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