Patient-specific autoantigen sample preparation and analysis using ProMTag

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Abstract

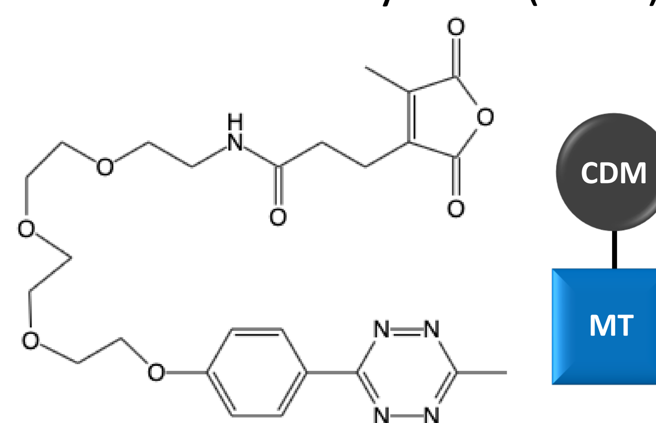
Autoimmune diseases affect >20 million people in the US today. Currently, disease-specific autoantibodies are thought to be the best biomarkers for diagnosis. Conventional immunoprecipitation methods have been used to identify autoantigens from the most common autoimmune diseases. However, these diseases account for only 6.5 million of the 20 million patients suffering from autoimmune diseases, leaving many without diagnoses until irreversible damage occurs. The remaining 13.5 million patients have >70 autoimmune disorders without well characterized autoantibodies. The state-of-the-art diagnostic test of these remaining diseases relies on gel electrophoresis of immunoprecipitated radiolabeled proteins, which cannot be identified by MS due to safety issues and the overwhelming presence of immunoglobulins.

We have created an immunoprecipitation method that uses serum from patients with any autoimmune disorder to identify patient-specific autoantigen proteins. This method uses a reversible click chemistry tag, called ProMTag. One end of the ProMTag forms a reversible, covalent bond with protein by coupling to lysines and amino termini. The other end of the ProMTag can form an irreversible, covalent bond with a solid bead support via a click chemistry, methyltetrazine-TCO, pairing. In this study, the proteins of cell lysates that contain potential autoantigens were labeled with ProMTag. The ProMTagged-proteins were exposed to patient antibodies bound to Protein A beads, thus capturing the ProMTagged autoantigens. All proteins were released from the Protein A beads, including ProMTagged- autoantigens and untagged-antibodies. The ProMTagged-autoantigens were subsequently coupled to TCO beads, and the untagged-antibodies were washed away. The linkage between the ProMTag and autoantigens was then reversed, yielding autoantigen proteins with greatly reduced antibody contamination ready for MS analysis. MS analysis successfully identified autoantigens from patient serums with rheumatoid arthritis. This autoimmune biomarker discovery method can accelerate sample testing for known autoantigens and facilitating rapid discovery of novel autoantigens for both diagnostic and predictive biomarkers.

ProMTag Workflow Key Components

ProMTag
Protein tagging

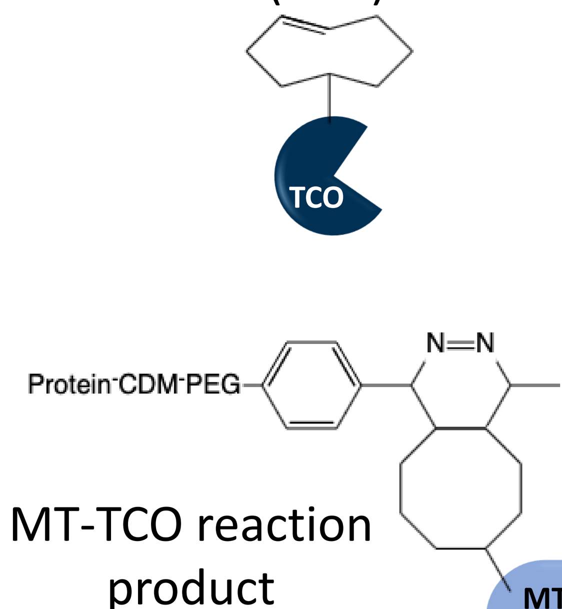
Reversible Protein Binding Reagent
Carboxyethyl-dialkyl-maleic Anhydride (CDM)



Irreversible Bead Binding Reagent
Methyltetrazine (MT)

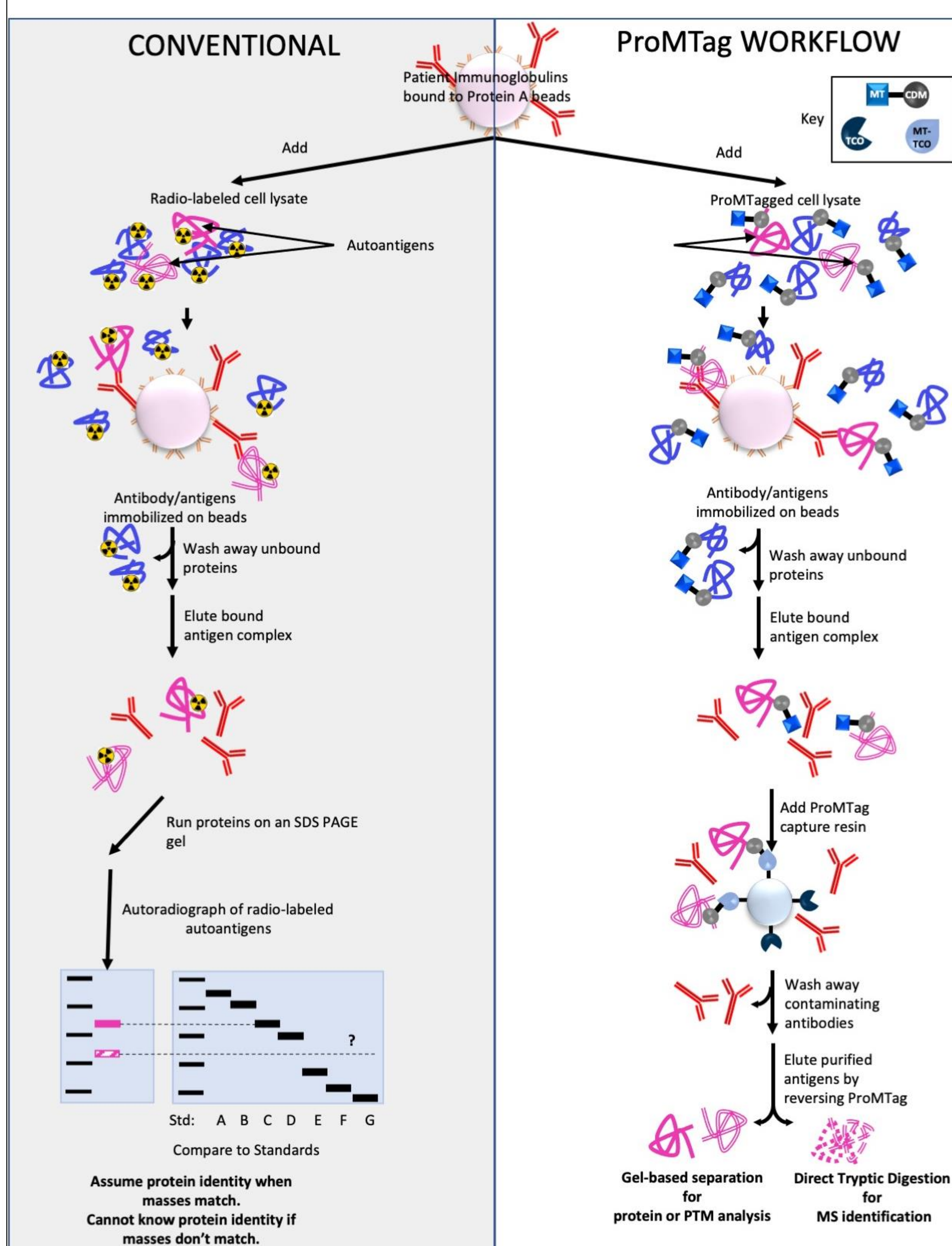
ProMTag Capture
TCO-bead

Trans-cyclooctene (TCO)

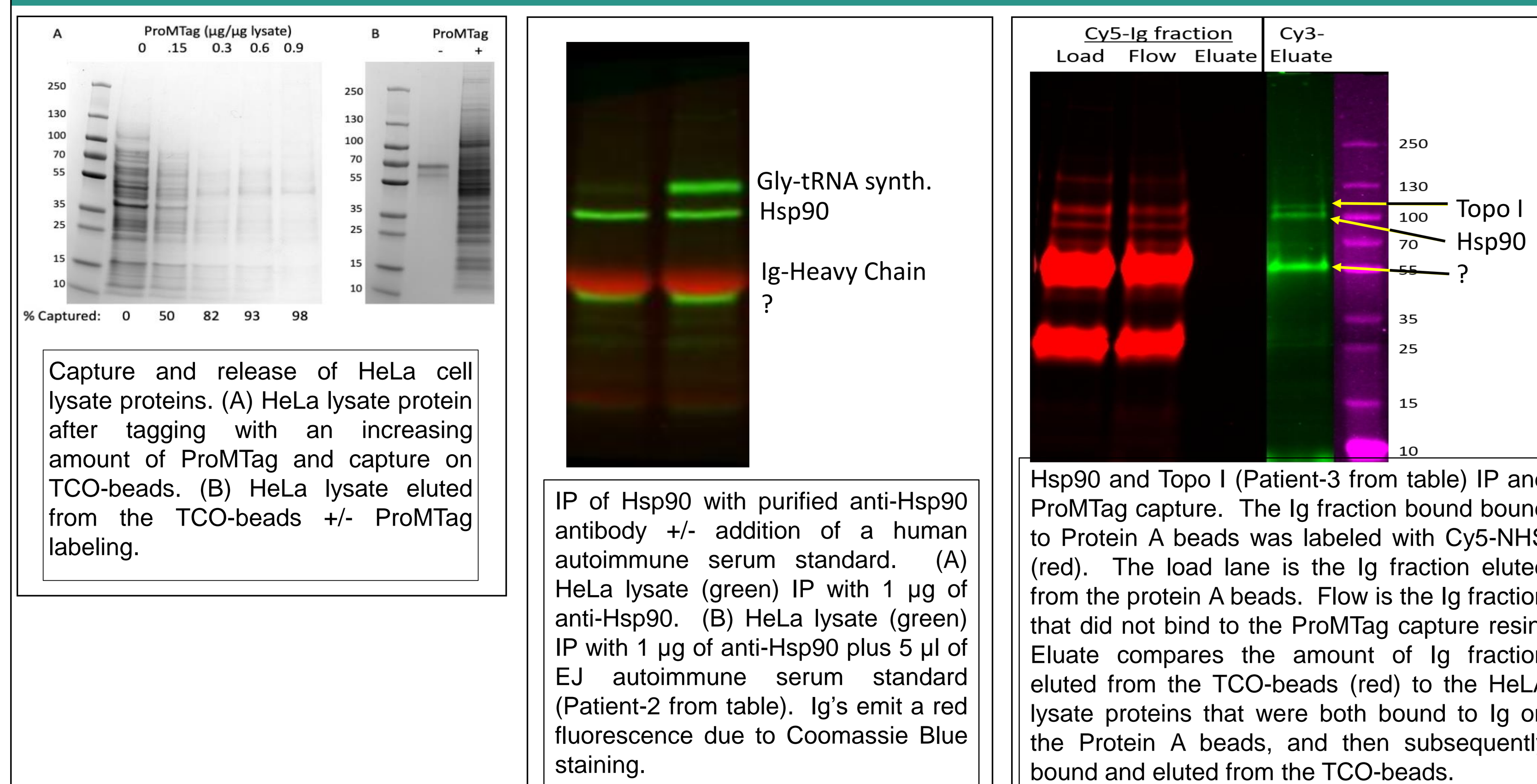


MT-TCO reaction product

Conventional vs ProMTag method for purification of autoantigens



Gel-based validation of the ProMTag immunoprecipitation workflow



Capture and release of HeLa cell lysate proteins. (A) HeLa lysate protein after tagging with an increasing amount of ProMTag and capture on TCO-beads. (B) HeLa lysate eluted from the TCO-beads +/- ProMTag labeling.

IP of Hsp90 with purified anti-Hsp90 antibody +/- addition of a human autoimmune serum standard. (A) HeLa lysate (green) IP with 1 µg of anti-Hsp90. (B) HeLa lysate (green) IP with 1 µg of anti-Hsp90 plus 5 µl of EJ autoimmune serum standard (Patient-2 from table). Ig's emit a red fluorescence due to Coomassie Blue staining.

Hsp90 and Topo I (Patient-3 from table) IP and ProMTag capture. The Ig fraction bound bound to Protein A beads was labeled with Cy5-NHS (red). The load lane is the Ig fraction eluted from the protein A beads. Flow is the Ig fraction that did not bind to the ProMTag capture resin. Eluate compares the amount of Ig fraction eluted from the TCO-beads (red) to the HeLA lysate proteins that were both bound to Ig on the Protein A beads, and then subsequently bound and eluted from the TCO-beads.

Control immunoprecipitation of K562 lysate with normal serum

Mass spectrometry results of negative control identify background proteins that are in the sample after the ProMTag IP workflow.

Normal Patient Serum Control

Description	Log Prob.	Total Intensity	# of spectra	# of unique peptides	Coverage %
Albumin	447.63	1.72E+09	197	69	78.82
Complement C3	443.94	9.78E+08	211	85	54.66
Vimentin	338.95	1.05E+09	135	50	74.03
Apolipoprotein B-100	224.01	1.62E+08	53	46	14.42
CD5 antigen-like	164.28	4.40E+08	72	22	72.05
Alpha-2-macroglobulin	84.98	8.10E+07	24	17	25.44
Polyadenylate-binding protein 1	81.26	1.39E+08	27	15	30.66
Tubulin beta chain	69.21	4.70E+07	13	8	32.43
Actin, cytoplasmic 2	56.92	1.37E+08	22	14	52.00
Complement C4-B	42.44	1.41E+07	5	4	3.10

Immunoprecipitation of K562 lysate with commercial anti-Hsp90 antibody

Mass spectrometry analysis of antigens after ProMTag IP workflow with antigen HSP90-alpha show successful identification of HSP90-alpha as expected.

Anti-HSP90 Rabit polyclonal - HSP90-alpha expected

Description	Log Prob.	Total Intensity	# of spectra	# of unique peptides	Coverage %
Isoform 2 of Heat shock protein HSP 90-alpha	844.83	4.26E+09	492	125	61.71
Vimentin	677.79	4.32E+09	428	110	92.92
Heat shock protein HSP 90-beta	468.76	1.11E+09	208	68	52.62
Actin, cytoplasmic 1	423.08	1.17E+09	179	56	77.07
Albumin	402.44	8.95E+08	139	55	70.94
Tubulin beta chain	386.28	7.92E+08	157	42	80.86
Fatty acid synthase	374.49	2.64E+08	75	53	34.81
Isoform 3 of Plectin	312.88	3.57E+08	95	64	18.64
Polyadenylate-binding protein 1	300.77	6.24E+08	117	62	66.51

Immunoprecipitation of K562 lysate with four characterized patient sera

Autoantibodies from patient sera with known autoantigens were immobilized on beads and used for IP of a ProMTag labeled protein lysate from K562 cells. IP'ed antigens were then sent for MS identification. In all four tests, the correct autoantigen was identified in the MS result. There were background proteins present in the samples (highlighted in yellow), however these proteins were also in the negative control and pure HSP90 antibody sample so we can rule them out as autoantigens.

Patient Serum 1 - Glycine tRNA ligase expected

Description	Log Prob.	Total Intensity	# of spectra	# of unique peptides	Coverage %
Apolipoprotein B-100	413.86	5.46E+08	108	74	22.16
Albumin	389.03	1.96E+09	193	62	70.61
Glycine--tRNA ligase	384.67	1.80E+09	167	63	69.69
Vimentin	232.97	5.40E+08	81	44	74.89
Complement C3	181.68	4.30E+08	78	43	32.05
CD5 antigen-like	160.45	9.13E+08	105	25	72.33
Complement C4-B	141.90	2.13E+08	26	20	19.04
Keratin, type II cytoskeletal 8	85.05	1.13E+08	19	11	28.16
Alpha-2-macroglobulin	79.41	9.09E+07	21	17	15.81

Patient Serum 2 - Topoisomerase 1 expected

Description	Log Prob.	Total Intensity	# of spectra	# of unique peptides	Coverage %
Complement C3	451.95	1.31E+09	190	97	62.42
Albumin	371.47	1.29E+09	147	60	72.09
Vimentin	293.19	7.91E+08	127	48	80.04
DNA topoisomerase 1	164.11	5.32E+08	80	44	44.31
Complement C4-B	147.37	1.46E+08	33	20	16.80
CD5 antigen-like	88.26	1.25E+08	23	13	62.82
Polyadenylate-binding protein 1	85.22	1.35E+08	30	16	28.30
Apolipoprotein B-100	83.80	1.17E+08	27	22	9.27
Cystatin-C	76.51	2.59E+08	48	16	72.60

Patient Serum 3 - Alanine tRNA ligase expected

Description	Log Prob.	Total Intensity	# of spectra	# of unique peptides	Coverage %
Alanine--tRNA ligase, cytoplasmic	679.11	1.53E+09	227	101	77.07
Albumin	504.07	2.26E+09	250	86	81.94
Vimentin	369.18	1.02E+09	132	57	82.40
Complement C3	354.78	6.60E+08	125	64	44.38
Apolipoprotein B-100	181.54	1.71E+08	50	40	13.87
Alpha-2-macroglobulin	174.88	2.25E+08	48	31	28.83
Complement C4-B	157.54	3.26E+08	56	28	17.37
CD5 antigen-like	126.47	3.54E+08	49	18	71.76
Actin, cytoplasmic 2	119.63	1.83E+08	34	19	60.53

Patient Serum 4 - Topoisomerase 1 expected

Description	Log Prob.	Total Intensity	# of spectra	# of unique peptides	Coverage %
Vimentin	369.29	2.18E+09	213	66	80.90
Albumin	350.79	1.66E+09	174	57	65.85
DNA topoisomerase 1	215.99	7.26E+08	99	47	49.41
CD5 antigen-like	190.55	9.19E+08	120	30	76.66
Complement C3	138.99	4.10E+08	63	44	35.84
Dihydrolipoyllysine-residue acetyltransferase	135.73	3.24E+08	59	27	46.21
Actin, cytoplasmic 2	122.25	1.58E+08	28	18	54.93
Alpha-2-macroglobulin	109.85	1.48E+08	30	24	27.68
Nuclear pore membrane glycoprotein 210	96.67	1.43E+08	32	25	23.85

- Expected protein IP target in pink; Common background proteins in yellow

Conclusions

The ProMTag immunoprecipitation method allowed for mass spectrometry of autoantigens immediately after immunoprecipitation, something that previously could not be done because of antibody contamination. Additionally, we correctly identified all known antigens in clinically relevant patient samples. However, there was residual background from common lysate and serum proteins. This background can be accounted for computationally or by optimizing the protocol's washing steps.

Acknowledgements

This work was supported by NSF SBIR Phase II Award 2036199. This work was also supported in part by NIH P30 CA124435 utilizing the Stanford Cancer Institute Proteomics/Mass Spectrometry Shared Resource at the Vincent Coates Foundation Mass Spectrometry Laboratory, Stanford University Mass Spectrometry (RRID:SCR_017801). We thank Ryan Leib and the Stanford University Mass Spectrometry Facility for their help with mass spectrometry analysis, data analysis, and statistical analysis.