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Laura Corrigan
Royal College of Surgeons in Ireland

Caroline Jefferies
Royal College of Surgeons in Ireland

T Clive Lee
Royal College of Surgeons in Ireland

Jacqueline Daly
Royal College of Surgeons in Ireland, jdaly@rcsi.ie

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Technical brief

Evaluation and optimization of IgY Spin Column technology in the depletion of abundant proteins from human serum

Laura Corrigan[1,2], Caroline Jefferies[3] , T. Clive Lee[2], and Jacqueline Daly[1,2]*

*Corresponding author: Laura Corrigan, Division of Biology, Department of Anatomy, Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2, Ireland; Tel: +35314022147; Fax: +3534022355; Email: lauracorrigan@rcsi.ie.

[1]Division of Biology, [2]Department of Anatomy, [3]Molecular and Cellular Therapeutics, Royal College of Surgeons in Ireland, Dublin, Ireland.

Abbreviations: 2DE: 2 Dimensional Gel Electrophoresis

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Abstract

While serum is frequently used in biomarker discovery, most of its total protein content is dominated by a small number of proteins, masking the presence of low abundance proteins, which may be candidate biomarkers of disease. Therefore, a serum depletion strategy is commonly implemented in order to remove abundant proteins, thereby increasing the number of proteins detected. Antibody-based Spin Columns offer a straightforward and cost-effective methodology of serum depletion. The Seppro® IgY Spin Columns used in this study bind 12 and 14 primate proteins respectively. 1D SDS PAGE and 2DE revealed a sub-optimal performance of the IgY Spin Columns. However, modification of the manufacturer's protocol; subjecting samples to two rounds of depletion, improved the number of proteins resolved by 2DE. Importantly, 2DE and MALDI-MS analysis revealed that no non-target proteins were removed from the human serum samples. With alteration of the manufacturer protocol the Seppro® IgY14 Spin Column can produce depleted serum with an increased number of spots resolved by 2DE compared to untreated serum.

Serum and plasma are commonly used in biomarker discovery studies [1]. However, a small number of proteins, such as albumin, dominate a large portion of the total protein content [2, 3]. The presence of these highly abundant proteins can mask the presence of lower abundance proteins among which are candidate biomarkers of disease. Therefore, the use of a serum depletion strategy to remove these abundant proteins is commonly employed [4-7].

Antibody-based depletion technologies present a method of depleting biofluids of abundant proteins in a specific manner. The Seppro® IgY system (Sigma) is used in this study. IgY antibodies are avian and display reduced non-specific binding compared with IgG antibodies [8]. IgY proteome partitioning technology was developed by Genway to remove 12 primate proteins from biofluids and the system has been subsequently developed to remove a further 2 proteins. This study aimed to assess the efficiency of depletion of abundant proteins from human serum by IgY Spin Column technology using 1D SDS PAGE, 2DE and MALDI-MS.

Abundant proteins were removed from healthy human serum using the Beckman Coulter IgY12 Spin Column (Phenomenexx). This contains IgY antibodies against albumin, IgG, fibrinogen, transferrin, IgA, IgM, apolipoprotein A-I and II, haptoglobin, α1 antitrypsin, α1 acidglycoprotein, and α2 macroglobulin. The Seppro® IgY14 Spin Column (Sigma) was also used. This removes two further proteins; apolipoprotein B and complement C3. 20µL serum was diluted with Dilution Buffer (supplied with columns) and filtered using Costar Spin-X centrifuge tubes 0.45µm pore size (Sigma). Serum was applied to the spin column and incubated for 15 minutes. Depleted serum was eluted by centrifugation for 30 seconds at 2,000 g. Depleted serum was acetone precipitated in 4 volumes of ice-cold acetone overnight at -20^{o}C. Spin Columns were then washed 3 times with Dilution Buffer. Abundant proteins were stripped from the Spin Columns by 4 steps of incubation in Stripping Buffer (supplied), followed by centrifugation for a total of no more than 15 minutes. Spin Columns were then immediately regenerated with Neutralising Buffer (supplied), incubated for 5 minutes followed by centrifugation. Samples were resuspended in sample buffer (9.5 M Urea; USB 2% CHAPS; USB, 0.8% Pharmalyte; GE Healthcare, 1% DTT; USB). 1D SDS PAGE was carried out on 4% acrylamide stacking gel, 10% acrylamide resolving gel using Laemmlli sample buffer (Sigma) and

run under 20mA current per gel in Laemmlli buffer. For 2DE, protein was passively rehydrated on IPG strips (GE Healthcare) pH interval 4-7 overnight. IEF was carried out for 9 kVolt hours (7cm IPG strips) or 80 kVolt hours (24cm IPG strips). IPG strips were equilibrated for SDS PAGE in 2 steps, 15 minutes each, using 6M urea (USB), 50 mM Tris HCl pH 8.8, 30% (v/v) glycerol (USB), 2% (w/v) SDS (USB) and adding 1% (w/v) DTT (USB) for the first equilibration and 2.5 % (w/v) iodoacetamide (USB) for the second equilibration. IPG strips were applied to 12% acrylamide gels and run at 20mA per gel (10x8 cm gels) or 1W per gel (24x20 cm gels) using Laemmlli buffer. Gels were stained with either Plus One Silver (GE Healthcare) or Coomassie (Invitrogen). Gels were scanned using the Biorad GS-800 Calibrated Densitometer scanner and analysed using Quantity One software. Coomassie stained 1D SDS PAGE bands were digested overnight with trypsin (Promega). MALDI-TOF and MALDI-TOF/TOF mass spectrometric analyses were carried out with a 4800 plus MALDI TOF/TOF Analyzer (Applied Biosystems). Peptide masses were acquired over a range from 800 to 4000 m/z, with a focus mass of 2000 m/z. MS spectra were summed from 2500 laser shots from an Nd: YAG laser operating at 355 nm and 200 Hz. An automated plate calibration was performed using five peptide standards (masses 900-2400 m/z; Applied Biosystems) in thirteen calibration wells. This plate calibration was used to update the instrument default mass calibration, which was applied to all MS and MS/MS spectra. A maximum of 12 precursors per sample well with a signal-to-noise ratio of >20 was automatically selected for subsequent fragmentation by collision induced dissociation. MS/MS spectra were summed from 4000 laser shots. Spectra were processed and analyzed by the Global Protein Server Workstation (Version 3.6, Applied Biosystems). This uses internally licensed MASCOT (Matrix Science) software for matching MS and MS/MS data against *in silico* protein digest databases. The MASCOT search parameters were set to run an all entries search against a UniProt knowledgebase (release 14.0, containing 392,667 entries) downloaded from the European Bioinformatics Institute homepage (http://ftp.ebi.ac.uk/pub/databases); allowing a single missed cleavage, two variable post-translational modifications (methionine oxidation and carbamidomethylation), a precursor tolerance of 100 ppm and MS/MS tolerance of 0.25 Da, and peptide charge of +1. Peptides were filtered for subsequent identification and analysis allowing a maximum

of top 10 ranked peptides using the Mowse scoring algorithm which calculates protein identification probability. A human specific database was filtered out from this UniProt knowledgebase.

Initial results following the manufacturer's instructions for both columns showed sub-optimal depletion (Figure 1A Lane 3, Figure 1E Lane 2). Further, the binding efficiency of the IgY12 Spin Column deteriorated over time (Figure 1B). To demonstrate that the IgY antibodies were saturated with bound protein we performed several cycles where no sample was applied to the IgY12 Columns. Figure 1C points to incomplete removal of protein from the Spin Column during the stripping phase of the previous depletion cycles (Lanes 1-4). Serum sample treated twice by IgY12 Spin Column (Figure 1D) shows reduced band of protein at the molecular weight of albumin compared with the same sample treated once. Reducing the starting volume of serum improves depletion using the IgY14 Spin Column (Figure 1E), however, 20µL depleted twice sample (Lane 6) shows the best depletion of abundant proteins. Initial 2DE experiments were carried out utilising 7cm IPG strips and mini gels; due to the low capacity of the IgY Spin Columns. A second application of a depleted 20µL sample to the column reveals a significantly different 2DE image (Figure 2A), indicative of the further removal of abundant proteins during the second cycle of depletion. A similar trend is observed using the IgY14 Spin Column in 2DE (Figure 2B). Further, resolution of higher molecular weight proteins is enhanced in the twice depleted sample compared with a single depletion. These results indicate that a reduction in the recommended starting volume of human serum produces an improvement in the resolution of proteins detected by 2DE. Moreover, treating serum samples twice provided further improvement in resolution of spots by 2DE. To assess non-specific removal of proteins by the IgY Spin Columns the bound fractions of both Spin Columns were analysed. Figures 2C and 2D show clean capture of abundant proteins and no removal of non-target proteins. MALDI-MS was used to confirm the identities of proteins bound by both IgY Spin Columns. No non-target proteins were identified by MALDI-MS (Table 1).

Spin Column technologies offer a relatively inexpensive and straightforward methodology for the removal of abundant proteins from biofluids. However, this study highlights insufficiencies with two generations of IgY Spin Columns. 1D SDS PAGE

and 2DE demonstrate that when following the supplied protocol, while there is a reduction in abundant proteins; albumin is visible in 20µL treated once by both IgY12 and IgY14 columns (Figure 1A Lane 3 and Figure 2B Lane 2). As the columns purport to remove a number of proteins, this inadequate removal of albumin is taken as indicative of overall sub-optimal performance of the column. Further, depletion deteriorated over time (Figure 1B) and significant amounts of albumin were eluted on cycles where no sample was loaded (Figure 1C). These issues were addressed with the re-application of eluted serum to further remove abundant proteins (Figure 1A Lane 2, Figure 1B Lane 6) and by the use of 'buffer only' cycles between samples to eradicate any bound protein not removed during the stripping phase of the depletion cycle. Optimization of the protocol produced much improved results by 1D SDS PAGE and 2DE (Figures 1 and 2). In Figure 2 mini-gel 2DE images are placed in parallel with large 2DE gel images. These initial mini-gels were carried out as the amount of protein eluted in the depleted serum fraction was relatively small. Figure 2A and 2B are intended as an illustration of a similar trend between the two columns. Both 2DE and MALDI-MS analyses of the human serum proteins bound by both columns revealed no non-target proteins. However, previous serum depletion studies have reported the removal of non-target proteins [9-11]. This study demonstrates that, with some alteration to the given protocol, suitably depleted serum can be produced by IgY Spin Columns in the context of a biomarker study.

References

1. Anderson, N.L., M. Polanski, R. Pieper, T. Gatlin, et al., *The human plasma proteome: a nonredundant list developed by combination of four separate sources.* Mol. Cell. Proteomics, 2004. **3**(4): p. 311-26.
2. Anderson, N.L. and N.G. Anderson, *The Human Plasma Proteome: History, Character, and Diagnostic Prospects.* Mol. Cell. Proteomics, 2002. **1**(11): p. 845-867.
3. Tirumalai, R.S., K.C. Chan, D.A. Prieto, H.J. Issaq, et al., *Characterization of the low molecular weight human serum proteome.* Mol. Cell. Proteomics, 2003. **2**(10): p. 1096-103.
4. Bjorhall, K., T. Miliotis, and P. Davidsson, *Comparison of different depletion strategies for improved resolution in proteomic analysis of human serum samples.* Proteomics, 2005. **5**(1): p. 307-17.
5. Plavina, T., E. Wakshull, W.S. Hancock, and M. Hincapie, *Combination of abundant protein depletion and multi-lectin affinity chromatography (M-LAC) for plasma protein biomarker discovery.* J Proteome Res, 2007. **6**(2): p. 662-71.
6. Andersen, J.D., K.L. Boylan, F.S. Xue, L.B. Anderson, et al., *Identification of candidate biomarkers in ovarian cancer serum by depletion of highly abundant proteins and differential in-gel electrophoresis.* Electrophoresis, 2010. **31**(4): p. 599-610.
7. Byrne, J.C., M.R. Downes, N. O'Donoghue, C. O'Keane, et al., *2D-DIGE as a Strategy To Identify Serum Markers for the Progression of Prostate Cancer.* J. Proteome Res. , 2008.
8. Huang, L., G. Harvie, J.S. Feitelson, K. Gramatikoff, et al., *Immunoaffinity separation of plasma proteins by IgY microbeads: meeting the needs of proteomic sample preparation and analysis.* Proteomics, 2005. **5**(13): p. 3314-28.
9. Granger, J., J. Siddiqui, S. Copeland, and D. Remick, *Albumin depletion of human plasma also removes low abundance proteins including the cytokines.* Proteomics, 2005. **5**(18): p. 4713-8.
10. Liu, T., W.J. Qian, H.M. Mottaz, M.A. Gritsenko, et al., *Evaluation of multiprotein immunoaffinity subtraction for plasma proteomics and candidate biomarker discovery using mass spectrometry.* Mol.Cell. Proteomics, 2006. **5**(11): p. 2167-74.
11. Gong, Y., X. Li, B. Yang, W. Ying, et al., *Different immunoaffinity fractionation strategies to characterize the human plasma proteome.* J. Proteome Res., 2006. **5**(6): p. 1379-87.

Figure 1. Arrows indicates band at molecular weight of albumin. A. 1D SDS PAGE of crude serum, proteins bound, and depleted serum eluted from the IgY12 Spin Column. Starting volume of serum was 20µL. 40µg protein was loaded in each lane. B. 1D SDS PAGE of crude serum and serum eluted from the IgY12 Spin Column after 50 uses (A and B Coomassie stained (Invitrogen)). C. 1D SDS PAGE of protein eluted from IgY12 Spin Column following 'buffer only' runs. Band shown is consistent with the molecular weight of albumin. C1=Spin Column 1 C2=Spin Column 2. (Silver stained (GE Healthcare)). D. 1D SDS PAGE of serum eluted from IgY12 Spin Column. 20µL serum was treated once and 20µL of an identical sample was treated twice. E. 1D SDS PAGE showing the effect of varying starting volume of serum using the IgY14 Spin Column. Lane 6 20 µL depleted twice shows the best depletion of abundant proteins. 10µg protein. (D and E Coomassie stained).

Figure 2. Silver stained 2DE of human serum samples following depletion by IgY Spin Columns. A. IgY12 treated samples; 10ug protein loaded on each gel, IPG strips pH 4-7 7cm. B. IgY14 Spin Column treated samples; 50µg protein loaded onto 24 cm IPG strips pH 4-7. LC=Light Chain. 2DE gel analysis of the bound protein from the IgY12 Spin Column (C) and the IgY14 Spin Column (D). Human serum was applied to the IgY Spin Columns, bound proteins stripped from the column and subject to 2DE, 24cm IPG strips ph4-7 and silver stained. Proteins were identified by eye through comparison with the 2DE of human plasma available on SWISS-2DPAGE Viewer on the ExPASy Proteomics Server. http://expasy.org/swiss-2dpage/viewer HC=Heavy Chain

Table 1. MALDI-MS results identifying proteins bound by IgY Spin Columns when human serum was applied, and the bound proteins subsequently stripped from the column. The bound proteins were subject to 1D SDS PAGE, Coomassie stained, bands of protein excised, trypsin digested and analysed by MALDI-MS. Table shows identifications with a confidence interval of >99%. A. IgY14 Spin Column: 7 proteins were identified by MALDI-MS, plus Ig kappa chain which may belong to the light chain of IgG (identified), IgM (identified) or IgA (not identified). B. IgY12 Spin Column: 9 proteins of the 12 listed were identified. Of the proteins not identified, fibrinogen was

not expected to be identified as it is present in plasma but not serum.*UniProtKB accession number

Figure 1.

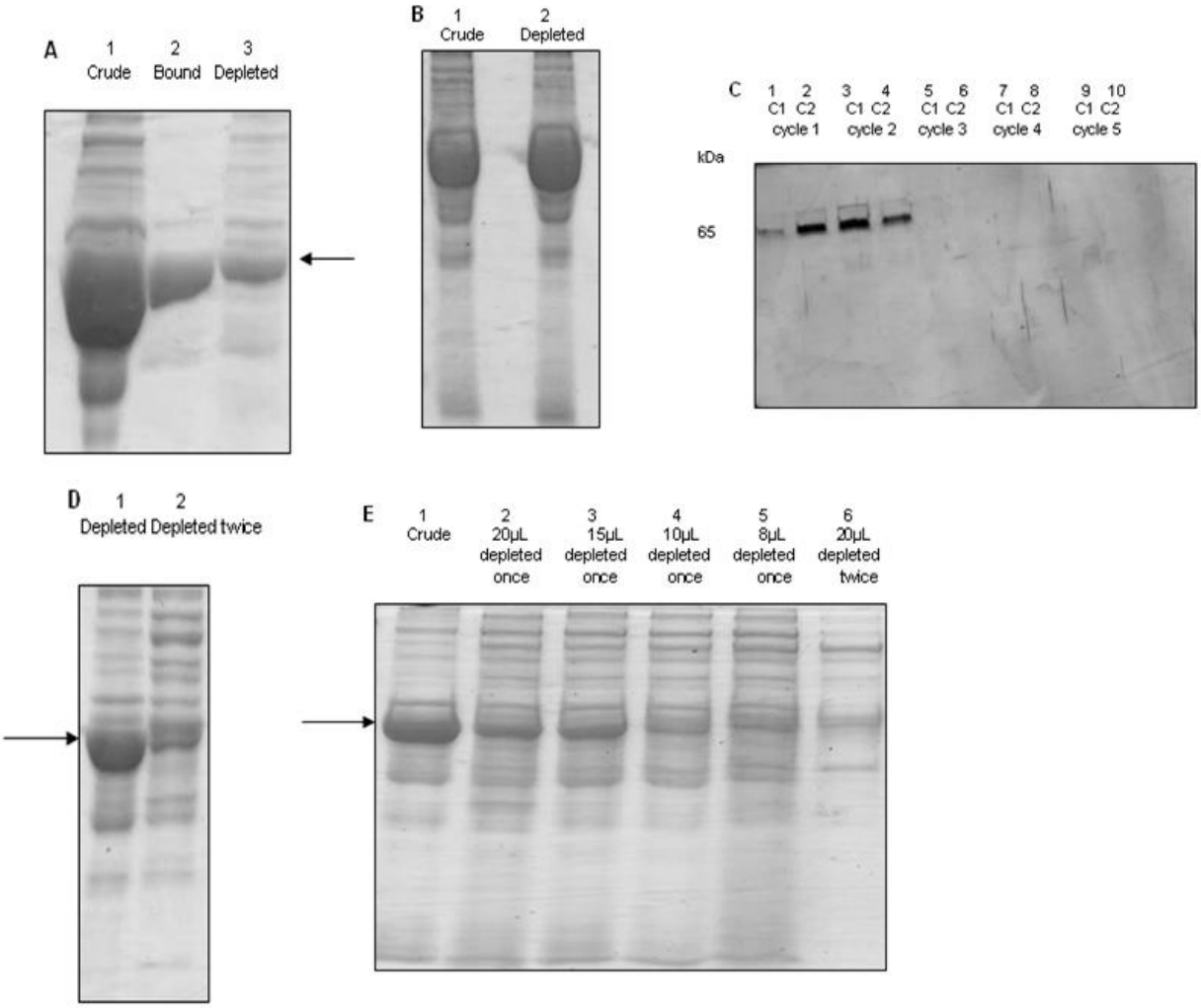
A
1 Crude
2 Bound
3 Depleted
B
1 Crude
2 Depleted
C
1 2 C1 C2 cycle 1
3 4 C1 C2 cycle 2
5 6 C1 C2 cycle 3
7 8 C1 C2 cycle 4
9 10 C1 C2 cycle 5
kDa
65
D
1 Depleted
2 Depleted twice
E
1 Crude
2 20µL depleted once
3 15µL depleted once
4 10µL depleted once
5 8µL depleted once
6 20µL depleted twice

Figure 2.

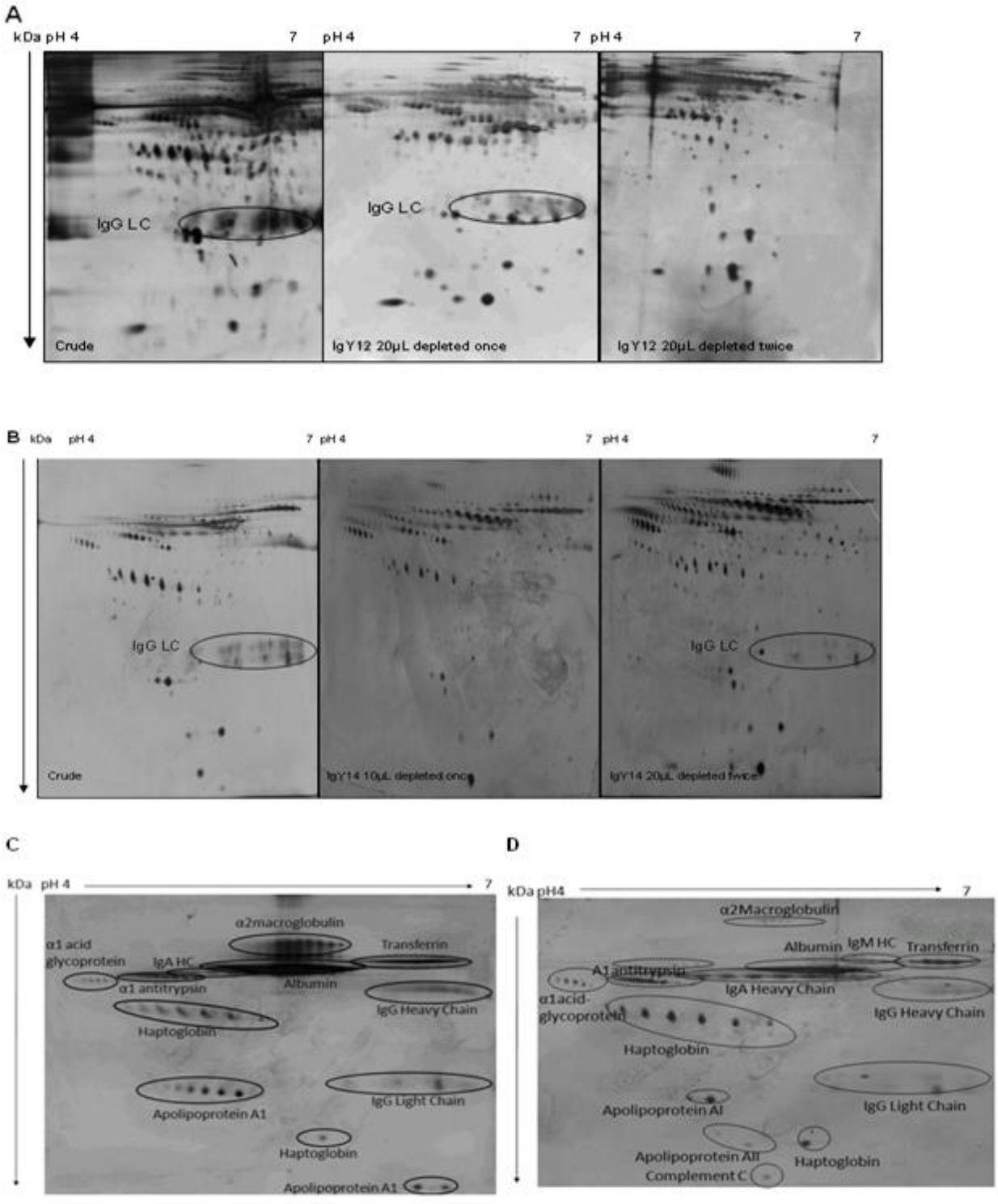
A
kDa pH 4
7 pH 4
7 pH 4
7
IgG LC
IgG LC
Crude
IgY12 20µL depleted once
IgY12 20µL depleted twice
B
kDa pH 4
7 pH 4
7 pH 4
7
IgG LC
IgG LC
Crude
IgY14 10µL depleted once
IgY14 20µL depleted twice
C
kDa pH 4
7
α2macroglobulin
α1 acid glycoprotein
IgA HC
Transferrin
α1 antitrypsin
Albumin
IgG Heavy Chain
Haptoglobin
Apolipoprotein A1
IgG Light Chain
Haptoglobin
Apolipoprotein A1
D
kDa pH4
7
α2Macroglobulin
Albumin
IgM HC
Transferrin
A1 antitrypsin
IgA Heavy Chain
α1 acid-glycoprotein
IgG Heavy Chain
Haptoglobin
Apolipoprotein AI
IgG Light Chain
Apolipoprotein AII
Haptoglobin
Complement C

Table 1.

A

Protein Name	Accession no.*	MW	Protein Score	Total Ion Score	No. matched peptides	Sequence Covered %
Serum Albumin	P02768	69321.5	178	154	11	21
Alpha-2-macroglobulin	P01023	163174.8	362	330	14	12
Ig gamma-1-chain C	P01857	36083.2	96	88	2	8
Ig gamma-2-chain C	P01859	35877.7	66	52	4	17
Transferrin	P02787	76999.6	179	148	11	25
Ig-mu-chain C	P01871	69321.5	83	58	7	17
Alpha-1-antitrypsin	P01009	36083.2	577	543	14	48
Haptoglobin	P00738	45176.6	192	133	11	27
Apolipoprotein A-1	P02647	30758.9	90	77	3	14
Ig alpha-1-chain C	P01876	37630.0	177	151	6	30

B

Protein Name	Accession no.*	MW	Protein Score	Total Ion Score	No. matched peptides	Sequence Covered %
Serum Albumin	P02768	69321.5	478	390	20	36
Alpha-2-macroglobulin	P01023	163174.8	242	208	15	13
Ig gamma-2-chain C	P01859	35877.7	127	95	7	40
Transferrin	P02787	76999.6	439	360	18	33
Ig mu chain C	P01871	69321.5	67	42	7	19
Alpha-1-antitrypsin	P01009	36083.2	288	246	9	27
Apolipoprotein A-1	P02647	30758.9	173	141	4	17
Ig kappa chain	P01834	11601.7	400	370	4	66

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