ProteomeLab IgY-12 High Capacity Proteome Partitioning Kits

Standard Operating Protocol

A24341AD

May 2012

Beckman Coulter, Inc.
250 S. Kraemer Blvd., Brea, CA 92821

Copyright, Licenses and Trademarks

Copyright

Licenses and Trademarks

ProteomeLab IgY-12 High Capacity Proteome Partitioning Kits

Introduction

The ProteomeLab IgY-12 High Capacity Proteome Partitioning kits are based on affinity columns using avian antibody (IgY)-antigen interactions and optimized buffers for sample loading, washing, eluting, and regenerating. They are specifically designed to remove twelve highly abundant proteins from human or primate biological fluids such as serum, plasma, and cerebral spinal fluid (CSF).

Improvements to the IgY immunoaffinity partitioning technology has allowed a doubling of the column capacity, which permits the researcher to load twice as much biological fluid per cycle on the columns, with a concommitant increase in the yield of partitioned protein.

This technology enables removal of albumin, IgG, α1-antitrypsin, IgA, IgM, transferrin, haptoglobin, α1-acid glycoprotein (orosomucoid), α2-macroglobulin, HDL (apolipoproteins A-I & A-II) and fibrinogen in a single step. The targeted highly abundant proteins are simultaneously removed by the immobilized specific IgY antibodies when complex biological samples are passed through the column. Selective immunoaffinity partitioning provides an enriched pool of lower abundant proteins for downstream proteomics analysis. Specific removal of twelve highly abundant proteins partitions up to 96% of total protein mass from human serum or plasma. The lower abundant proteins in the flow-through fractions and the highly abundant proteins in the bound/eluted fractions can be collected and further fractionated with the ProteomeLab PF 2D System or other fractionation or analytical methods. The technology uses physiological buffers for binding and washing, and avoids urea and other chaotropic agents for elution that can precipitate at low temperature. The enriched proteome, which includes medium and low abundance proteins, is the primary target for discovery and validation of biomarkers, toxicoproteomics, animal disease models, drug targets, and therapeutic proteins and antibodies.

ProteomeLab IgY-12 High Capacity Requirements and Yields

The ProteomeLab IgY-12 SC, IgY-12 LC2, and IgY-12 LC10 High Capacity Proteome Partitioning kits are selected based on the following:

- the volume of human or other primate biological fluids needed to yield the target quantity of partitioned protein for subsequent analysis
- the sample throughput requirements
- the availability of liquid chromatography equipment

The IgY-12 High Capacity SC Spin Column kit is based on centrifugation and has a capacity of 20 µL human or primate serum or plasma per spin column cycle. The expected yield of a sample partitioned of the 12 highly abundant proteins is about 160 µg. We recommend the use of the ProteomeLab SP Sample Preparation System with the IgY-12 High Capacity SC kit.

The IgY-12 High Capacity LC2 column kit requires liquid chromatography equipment and has a capacity of 50 µL human or primate serum or plasma per LC column cycle. The expected yield of a sample partitioned of the 12 highly abundant proteins is about 400 µg. We recommend the use of the ProteomeLab PPS Proteome Partitioning System with the IgY-12 High Capacity LC2 column kit.

The IgY-12 High Capacity LC10 column kit also requires liquid chromatography equipment and has a capacity of 250 µL human or primate serum or plasma per LC column cycle. The expected yield of a sample partitioned of the 12 highly abundant proteins is about 2 mg. Samples can be pooled for ProteomeLab PF 2D (recommended 1 - 5 mg) or other fractionation methods and can be analyzed by 2D electrophoresis or LC-MS methods. We recommend the use of the ProteomeLab PPS Proteome Partitioning System with the IgY-12 High Capacity LC10 column kit.

Under proper conditions of sample preparation and affinity chromatography, each column is capable of 100 cycles before replacement is needed. There is sufficient buffer supplied for 100 cycles per column.

Additional Proteome Partitioning Kits

ProteomeLab IgY Proteome Partitioning kits in SC, LC2, and LC10 formats containing IgY antibodies directed against multiple abundant proteins are also available for:

- IgY-R7 for rodent samples partitioning seven highly abundant proteins

ProteomeLab IgY Proteome Partitioning Spin Column kits containing IgY antibodies directed against single abundant proteins are also available for:

- Anti-HSA (human serum albumin) High Capacity
- Anti-IgG-Fc
- Anti-transferrin
- Anti-fibrinogen
- Anti-HDL
- Anti-BSA (bovine serum albumin)
- Anti-RSA (rat serum albumin)

IgY-12 High Capacity Spin Column

Reorder number: A24618

Observed Capacity*: 20 µL human or other primate serum or plasma (per run)

Kit Contents	Product Description
IgY-12 High Capacity SC 2 x 1.2 mL IgY-12 High Capacity Microbeads packed in a spin column	Spin Column consisting of anti-human serum albumin, anti-IgG, anti-fibrinogen, anti-transferrin, anti-IgA, anti-IgM, anti-HDL (anti-apo A-I and anti-apo A-II), anti-haptoglobin, anti-α1-antitrypsin, anti-α1-acid glycoprotein and anti-α2-macroglobulin. Affinity-purified anti-IgY antibodies are covalently conjugated through their Fc portion to 60 µm polymeric microbeads as a 50% resin in Dilution Buffer with 0.02% Sodium Azide.
6 empty spin columns 4 spin column end caps	Used for transferring resin after every 25 cycles and for sealing spin column tips.
1x Dilution Buffer (4 x 180 mL)	Tris Buffered Saline: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl. For sample dilution, washing and equilibrating column, and rinsing pipette tips during resin transfer.
1x Stripping Buffer (2 x 120 mL)	0.1 M Glycine-HCl, pH 2.5 for stripping off bound proteins from column.
10x Neutralization Buffer (2 x 20 mL)	1 M Tris-HCl, pH 8.0 for neutralizing column and eluted proteins.
1000 Collection tubes	2 mL collection tube vials.

*The IgY Microbeads are reusable for 100 times under proper conditions, including transfer of resin into a fresh spin column after every 25 uses.

IMPORTANT: The Neutralization buffer is shipped as 10x buffer. Use undiluted buffer to neutralize eluted proteins. Dilute 10 mL of this buffer to 1x with 90 mL of HPLC grade water prior to use for neutralizing the spin column.

ProteomeLab Spin Column Common Characteristics

Antibody Produced From:	Chicken, polyclonal. Antigen affinity-purified
Column Storage Buffer:	Dilution Buffer with 0.02% sodium azide
Column Storage Instruction:	2-8°C **Do not freeze.**
Recommended Application:	Plasma or serum protein separation, immunoprecipitation.

Materials Required but Not Supplied in this Kit

- ProteomeLab SP Sample Preparation System including one F2402H fixed-angle rotor
- End-to-end Rotator (e.g., Glas-Col Catalog No. 099A-RD4512)
- Pipette and pipette tips (1-200 µL and 200-1000 µL)
- Column Storage Dilution Buffer with 0.02% (w/v) Sodium Azide
- Microcon YM-3 Centrifugal Filter Device (Millipore, Catalog No. 42420 for the 8 pack; Catalog No. 42403 for the 24 pack; Catalog No. 42404 for the 100 pack)
- HPLC grade water
- Gloves and protective eyewear

Safety Issues

When preparing biological samples using ProteomeLab Columns, follow general guidelines for handling biological material and wear the appropriate protective attire.

Methods

Immunocapture of 12 Abundant Serum/Plasma Proteins

1. Dilute a 20 µL sample in 480 µL Dilution Buffer to get a final volume of 500 µL (1:25 dilution).
2. For the first use, snap off the tip from the column and place the column in a 2 mL centrifuge collection tube.
3. Before every run, ensure all buffer bottles are free of turbidity and precipitation. Replace them if present.
4. Centrifuge the column for 30 sec. at 400 x *g* to obtain dried beads.
5. Place the end cap on the column tip. Immediately add 0.5 mL diluted sample to the dried beads in the column. Seal the column with the top snap cap.
6. Mix the beads and the sample completely by inverting and shaking the column.
7. Place the sample on an end-to-end rotator and incubate it at room temperature for 15 min.
8. Invert the column. Remove the end cap and place the column in a 2 mL centrifuge collection tube. Centrifuge for 30 seconds at 400 x *g*. Collect the flow-through (partitioned) sample for further analysis.
9. To obtain maximum yields of flow-through samples, an optional wash step can be applied. Add 0.5 mL Dilution Buffer to the beads. Mix beads and buffer completely by inverting and shaking the column. Centrifuge for 30 seconds at 400 x *g*. Collect and combine with the flow-through sample from step 8 for further analysis.
10. Concentrate the sample using the Microcon YM-3 centrifugal filter device. Refer to the protein concentration instructions as provided by the manufacturer.

Stripping of Bound Proteins

1. To remove proteins non-specifically bound to microbeads, wash the beads with Dilution Buffer, a total of 3 times. For each wash, always first insert the end cap, and then add 0.5 mL Dilution Buffer and seal the column with top snap cap. Mix the beads and buffer completely by inverting and shaking the column, remove the end cap while inverting the column and place it in a 2 mL centrifuge collection tube. Centrifuge for 30 seconds at 400 x *g* and save the flow-through for further analysis.
2. Strip off bound proteins from beads using Stripping Buffer a total of 2 times. For each elution, place the end cap to the column first after centrifugation, then add 0.5 mL Stripping Buffer and seal the column with top snap cap. Mix the beads and buffer completely by inverting and shaking the column, incubate at room temperature for 3 min., remove the end cap while holding the column upside down and place it in a 2 mL centrifuge collection tube. Centrifuge for 30 seconds at 400 x *g* and collect the eluant.

   It is crucial to neutralize the beads IMMEDIATELY for column stability.
   See "Spin Column Regeneration" on page 7.

3. Pool two eluted samples (total ~1 mL) and neutralize with 100 µL of 10x Neutralization Buffer. Samples can be further concentrated using the centrifugal filter device to achieve the desired concentration and volume. Refer to the protein concentration instructions provided by the manufacturer.

Spin Column Regeneration

1. To regenerate ProteomeLab beads after stripping bound proteins as described above, **immediately** neutralize beads with 0.6 mL of 1:10 diluted Neutralization Buffer. Mix beads and buffer completely by inverting and shaking the column. Incubate at room temperature for 5 min.
2. Spin down beads in the column for 30 seconds at 400 x *g*.
3. Resuspend beads in 0.5 mL Dilution Buffer. Beads are ready for storage at 4°C or the next separation for up to 7 days. For storage of regenerated beads for more than 7 days, it is suggested that the Dilution Buffer contain 0.02% (w/v) sodium azide. In either case, seal the column with an end cap prior to storage.
4. Keep careful track of the number of times each spin column is used. After cycles 25, 50, and 75, transfer resuspended resin (1.2 mL) with a disposable 1000 µL pipette tip into a fresh empty spin column.
5. To efficiently transfer beads to a new spin column after each set of 25 runs, we suggest re-suspending the beads with 500 µL Dilution Buffer, then transferring them to a new spin column. Centrifuge the new column in a collection tube for 30 seconds at 400 x *g*, rinse the old column with 500 µL Dilution Buffer, and transfer with the same tip to the new spin column to completely recover residual microbeads.

Troubleshooting

Symptom	Possible Cause	Corrective Action
Reduction in protein binding capacity	Column may have not been regenerated well enough from previous runs.	Elute bound proteins with Stripping Buffer for 2 additional column volumes, and then immediately neutralize and re-equilibrate the column with 1x Neutralization Buffer and Dilution Buffer.
	Buffers contaminated with bacteria or fungi.	Carefully check for signs of biological growth and precipitates in the Buffers. Replace with fresh Buffers for optimized column performance.
	Diminished resin bed volume due to serial transfer into new spin columns.	After each set of 25 runs, it is suggested to re-suspend the beads with 500 µL Dilution Buffer, then transfer them to a new spin column. Centrifuge the new column in a collection tube for 30 seconds at 400 x *g*, rinse the old column with 500 µL Dilution Buffer, and transfer with the same tip to the new spin column to completely recover residual microbeads.

Additional Technical Support

In North America:

Contact Beckman Coulter Technical Support
1.800.742.2345
proteomelab@beckman.com

Worldwide:

Contact your Beckman Coulter Technical Support Associate.

Expected Performance

Affinity Separation of 12 Abundant Proteins from Human Serum

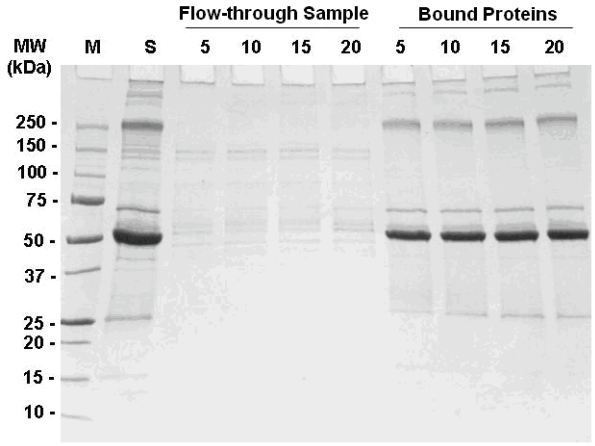


Figure 1: Recyclability of ProteomeLab IgY-12 spin column. The ProteomeLab IgY-12 spin column was used to separate 10 µL human serum proteins 20 times in succession. Selected samples were analyzed by 4-20% SDS-PAGE under non-reducing conditions. M, Molecular weight marker; S, Unfractionated human serum; 5, 10, 15 and 20, Samples (Flow-through or bound proteins) from the 5th, 10th, 15th and 20th cycle of the same ProteomeLab IgY-12 spin column.

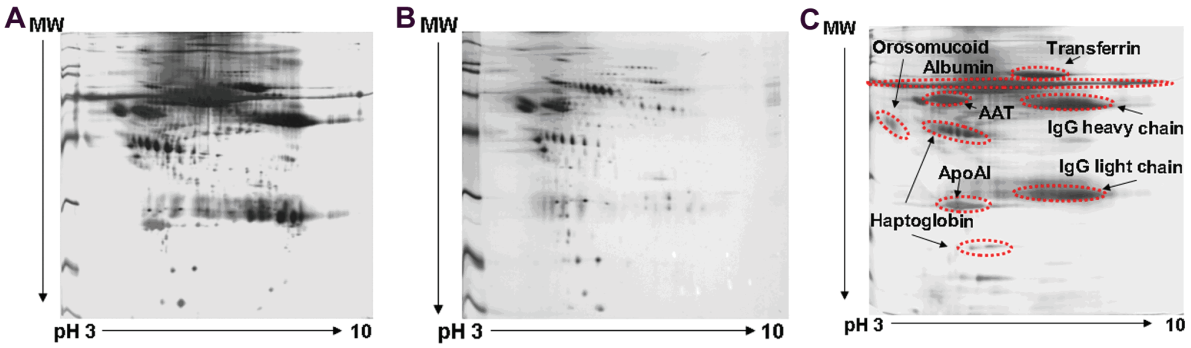


Figure 2: 2DE protein fingerprinting of human serum before and after ProteomeLab IgY-12 partitioning. (A) Unfractionated human serum proteins before ProteomeLab IgY-12 treatment, (B) Human serum protein fraction after ProteomeLab IgY-12 treatment partitioned of the 12 most abundant proteins. (C) Proteins eluted from ProteomeLab IgY-12 column. 100 µg of protein was loaded on each gel.

ProteomeLab IgY-12 High Capacity LC2 Proteome Partitioning Kit

Using these Instructions

Both of the ProteomeLab LC Proteome Partitioning Kits have specific measurements, requirements, and protocols. After noting the Protocol for this kit, See "ProteomeLab LC Partitioning Kit General Documentation" on page 23, for Troubleshooting and Recommendations.

Reorder number: A24346

Observed Capacity*: 50 µL human or other primate serum or plasma (per run)

Kit Contents	Product Description
IgY-12 High Capacity LC2 6.4 x 63 mm, affinity column	LC Column consisting of anti-human serum albumin, anti-IgG, anti-fibrinogen, anti-transferrin, anti-IgA, anti-IgM, anti-HDL (anti-apo A-I and anti-apo A-II), anti-haptoglobin, anti-α1-antitrypsin, anti-α1-acid glycoprotein, and anti-α2-macroglobulin. Affinity-purified anti-IgY antibodies are covalently conjugated through their Fc portion to 60 µm polymeric microbeads as a 50% resin in Dilution Buffer with 0.02% Sodium Azide.
10x Dilution Buffer (200 mL)	Tris Buffered Saline: 100 mM Tris-HCl, pH 7.4, 1.5 M NaCl. For sample dilution, washing and equilibrating column.
10x Stripping Buffer (180 mL)	1 M Glycine-HCl, pH 2.5 for stripping off bound proteins from column.
10x Neutralization Buffer (2 x 80 mL)	1 M Tris-HCl, pH 8.0 for neutralizing column and eluted proteins.
100 Spin Filters, 0.45 µm	For sample clean up before loading column to remove sample particulates and extend column life.

*The IgY Microbeads are reusable for 100 times under proper conditions.

IMPORTANT: All buffers are shipped as 10x buffer. Dilute buffers to 1x with HPLC grade water prior to use. For neutralization of eluted proteins, retain 50 mL of undiluted 10x Neutralization Buffer.

ProteomeLab LC Column Common Characteristics

Antibody Produced From:	Chicken, polyclonal. Antigen affinity-purified
Column Storage Buffer:	Dilution Buffer with 0.02% sodium azide
Column Storage Instruction:	2-8°C **Do not freeze.**
Recommended Application:	Plasma or serum protein separation, immunoprecipitation.

Column Specifications

Column Name:	IgY-12 High Capacity LC2
Column Size:	6.4 x 63 mm (2 mL bed volume)
Column body materials:	Polycarbonate column cylinder, Polyethylene frit, Tefzel caps, Buna-N-rubber O rings, Delrin nut fittings, ETFE ferrules, and Teflon PFA tubing.
Column capacity:	50 µL human or other primate serum/plasma
Maximum pressure:	100 PSI (6 bar)
Operating temperature:	18-25°C
Column packing material:	Antibody-modified resin
Immobilized ligands:	Affinity-purified IgY polyclonal antibodies to human albumin, IgG, α1-antitrypsin, IgA, IgM, transferrin and haptoglobin, α1-acid glycoprotein (orosomucoid), α2-macroglobulin, HDL (apolipoproteins A-I & A-II), and fibrinogen.
Flow rate range:	0.1-1 mL/min

Materials Required but Not Supplied in this Kit

- ProteomeLab SP Sample Preparation System, including one F2402H Fixed Angle rotor and one SX4250 Swinging Bucket rotor with Adapter PN 392257 for a 15 mL conical tube
- ProteomeLab PPS Proteome Partitioning System
- Pipette and pipette tips (1-200 µL and 200-1000 µL)
- Column Storage Dilution Buffer with 0.02% (w/v) Sodium Azide
- Amicon Ultra-4 Centrifugal Filter Device (Millipore, Catalog No. UFC8 005 08 for the 8 pack, or Catalog No. UFC8 005 24 for the 24 pack)
- HPLC grade water
- 10 mL polypropylene tubes for fraction collection
- 1000 mL graduated cylinder
- Gloves and protective eyewear

Safety Issues

When preparing biological samples using ProteomeLab Columns, follow general guidelines for handling biological materials and wear the appropriate protective attire.

Methods

Protocol for 6.4 x 63 mm LC2 High Capacity Column (Column capacity: 50 µL human serum/plasma)

1. Dilute 100 mL of each stock 10x buffer with 900 mL HPLC grade water.
2. Transfer each 1x buffer to a separate 1 liter solvent bottle.
3. Before every run, ensure all buffer bottles are free of turbidity and precipitation. Replace them if these conditions are present.
4. Prime the solvent lines with the three 1x diluted buffers (Dilution, Stripping, and Neutralization) according to the ProteomeLab PPS Operators Manual or the instructions of other chromatographic systems.
5. Start the 32 Karat software and select the `IgYLC2.met` method file on the ProteomeLab PPS according to the User's Guide, or program the chromatographic system, using the instructions found in the programming guidelines.
6. Set up the LC timetable. See "Appendix A: IgY-12 High Capacity LC2" on page 25 for guidelines on non-ProteomeLab PPS Systems.
7. Attach the column and set the injector to the inject position.

8. Before the first run of the day, after removing the column from the refrigerator, equilibrate with 1x Dilution Buffer for 20 min. at a flow rate of 1.0 mL/min. at room temperature to ensure a flat baseline.
   - The first time the column is used and after temperature equilibration, perform two complete buffer runs without injecting a sample.
9. Dilute the 50 µL sample in 75 µL of 1x Dilution Buffer to get a final volume of 125 µL (1 to 2.5 dilution).
10. Remove any sample particulates using a 0.45 µm spin filter. Centrifuge for 1 minute at 9200 x *g*.
11. Inject 125 µL of the diluted sample and start the separation method.
12. Collect flow-through and eluted fractions. Add 1/10 volume of 10x Neutralization Buffer (0.35-0.45 mL) to the eluted fraction (3.5-4.5 mL). Store both fractions at -70°C, if they are not analyzed immediately.
13. If the column is not going to be reused within 7 days, equilibrate the column with 1x Dilution Buffer that contains 0.02% (w/v) sodium azide. Start the 32 Karat software and select `IgYLC2_store.met` method file on the ProteomeLab PPS System, or pump the 1x Dilution Buffer with 0.02% sodium azide at a flow rate of 1.0 mL/min. for 10 min. through the column on other chromatographic systems. Store the column at 2-8°C in a refrigerator.

    DO NOT FREEZE THE COLUMN.

14. Concentrate the flow through and bound protein samples with the Amicon Ultra-4 centrifugal filter unit, 5,000 molecular weight limit according to the protein concentration instructions as provided by the manufacturer.

NOTE: For further fractionation using the ProteomeLab PF 2D, continue with the next 2 steps.

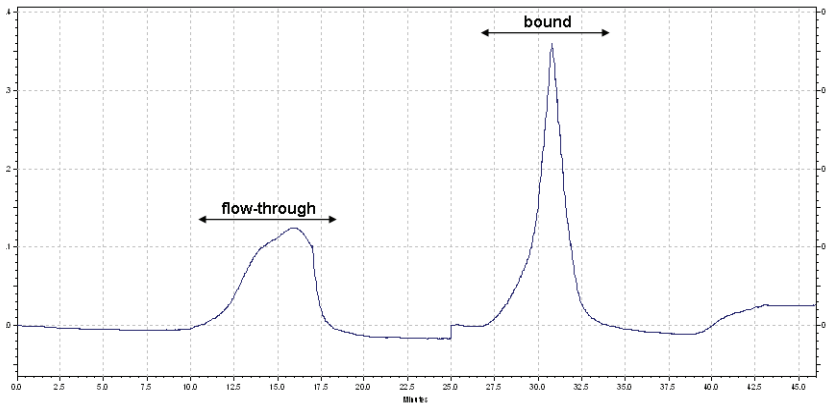
15. After concentrating the flow-through and/or the stripped protein sample to a minimum volume, add ProteomeLab PF 2D Start Buffer to a final volume of 4.0 mL and concentrate again.
16. Finally, add a volume of Start Buffer to bring the final volume to 2.0 mL for injection on the ProteomeLab PF 2D.

It is suggested that 1-5 mg of the protein be injected on the ProteomeLab PF 2D, which will require 5 to 25 cycles of the LC2 column.

Expected Performance

Recyclability of ProteomeLab IgY-12 LC2

A



B

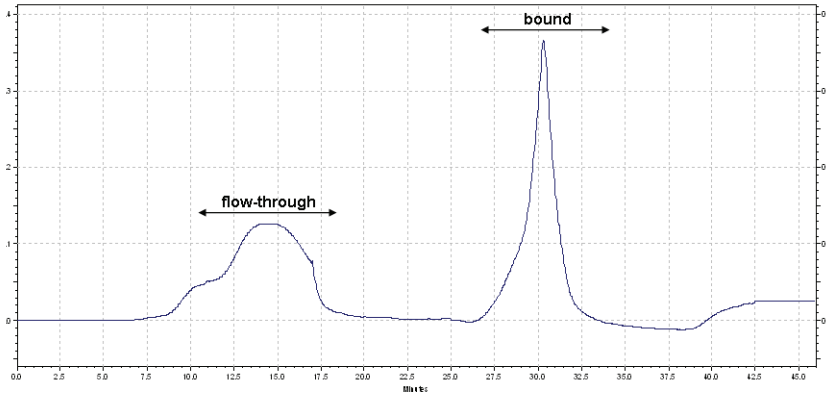


Figure 3: Recyclability of ProteomeLab IgY-12 LC2. The ProteomeLab IgY-12 LC2 column was used to partition 25 µL primate serum proteins 105 times in succession. The chromatograms shown are from run 1 (A) and run 105 (B). Note the consistency of the protein profiles. The A_{280} absorbance of the flow-through peak appears to be less than the bound peak. The expected volume of the flow-through fraction is 2.5 - 3.0 mL. The expected volume of the bound fraction is 3.5 - 4.5 mL.

ProteomeLab IgY-12 High Capacity LC10 Proteome Partitioning Kit

Using these Instructions

Both of the ProteomeLab LC Proteome Partitioning Kits have specific measurements, requirements, and protocols. After noting the Protocol for this kit, See "ProteomeLab LC Partitioning Kit General Documentation" on page 23, for Troubleshooting and Recommendations.

Reorder number: A24355

Observed Capacity*: 250 µL human or other primate serum or plasma (per run)

Kit Contents	Product Description
IgY-12 High Capacity LC10 12.7 x 79.0 mm, affinity column	LC Column consisting of anti-human serum albumin, anti-IgG, anti-fibrinogen, anti-transferrin, anti-IgA, anti-IgM, anti-HDL (anti-apo A-I and anti-apo A-II), anti-haptoglobin, anti-α1-antitrypsin, anti-α1-acid glycoprotein, and anti-α2-macroglobulin. Affinity-purified anti-IgY antibodies are covalently conjugated through their Fc portion to 60 µm polymeric microbeads as a 50% resin in Dilution Buffer with 0.02% Sodium Azide.
10x Dilution Buffer (3 x 220 mL)	Tris Buffered Saline: 100 mM Tris-HCl, pH 7.4, 1.5 M NaCl. For sample dilution, washing and equilibrating column.
10x Stripping Buffer (2 x 200 mL)	1 M Glycine-HCl, pH 2.5 for stripping off bound proteins from column.
10x Neutralization Buffer (2 x 250 mL)	1 M Tris-HCl, pH 8.0 for neutralizing column and bound proteins.
100 Spin filters, 0.45 µm	For sample cleanup before loading column to remove sample particulates and extend the column life.

* The IgY Microbeads are reusable for 100 times under proper conditions.

IMPORTANT: All buffers are shipped as 10x buffer. Dilute buffers to 1x with HPLC grade water prior to use. For neutralization of eluted proteins, retain 200 mL of undiluted 10x Neutralization Buffer.

ProteomeLab LC Column Common Characteristics

Antibody Produced From:	Chicken, polyclonal. Antigen affinity-purified.
Column Storage Buffer:	Dilution Buffer with 0.02% sodium azide
Column Storage Instruction:	2-8°C **Do not freeze.**
Recommended Application:	Plasma or serum protein separation, immunoprecipitation.

Column Specifications

Column Name:	IgY-12 High Capacity LC10
Column Size:	12.7 x 79.0 mm (10 mL bed volume)
Column body materials:	Polycarbonate column cylinder, Polyethylene frit, Tefzel caps, Buna-N-rubber O rings, Delrin nut fittings, ETFE ferrules, and Teflon PFA tubing.
Column capacity:	250 µL human or other primate serum/plasma
Maximum pressure:	100 PSI (6.0 bar)
Operating temperature:	18-25°C
Column packing material:	Antibody-modified resin
Immobilized ligands:	Affinity-purified IgY polyclonal antibodies to human albumin, IgG, α1-antitrypsin, IgA, IgM, transferrin and haptoglobin, α1-acid glycoprotein (orosomucoid), α2-macroglobulin, HDL (apolipoproteins A-I & A-II), and fibrinogen.
Flow rate range:	0.5-2.0 mL/min

Materials Required but Not Supplied in this Kit

- ProteomeLab SP Sample Preparation System, including one F2402H Fixed Angle rotor and one SX4250 Swinging Bucket rotor with Adapter PN 392258 for 50 mL conical tube
- ProteomeLab PPS Proteome Partitioning System
- Pipette and pipette tips (1-200 µL and 200-1000 µL)
- Column Storage Dilution Buffer with 0.02% (w/v) sodium azide
- Amicon Ultra-15 Centrifugal Filter Device (Millipore, Catalog No. UFC9 005 08 for the 8 pack, Catalog No. UFC9 005 24 for the 24 pack, or Catalog No. UFC9 005 96 for the 96 pack)
- HPLC grade water
- 50 mL polypropylene tubes for fraction collection
- 1000 mL graduated cylinder
- Gloves and protective eyewear

Safety Issues

When preparing biological samples using ProteomeLab Columns, follow general guidelines for handling biological materials and wear the appropriate protective attire.

Methods

Protocol for 12.7 x 79.0 mm LC10 High Capacity Column (Column capacity: 250 µL human serum/plasma)

1. Dilute 100 mL of each stock 10x buffer with 900 mL HPLC grade water.
2. Transfer each 1x buffer to a separate 1 liter solvent bottle.
3. Before every run, ensure all buffer bottles are free of turbidity and precipitation. Replace them if these conditions are present.
4. Prime the solvent lines with the three 1x diluted buffers (Dilution, Stripping, and Neutralization) according to the ProteomeLab PPS Operators Manual or as instructed for other chromatographic systems.
5. Start the 32 Karat software and select the `IgYLC10.met` method file on the ProteomeLab PPS according to the User's Guide, or program the chromatographic system, using the instructions found in the programming guidelines.
6. Set up LC timetable. See "Appendix B: IgY-12 High Capacity LC10" on page 26 for guidelines on non-ProteomeLab PPS Systems.
7. Attach the column and set the injector to the inject position.

8. Before the first run of the day, after removing the column from the refrigerator, equilibrate with 1x Dilution Buffer for 30 min. at a flow rate of 2.0 mL/min. at room temperature to ensure a flat baseline.
   - The first time the column is used and after temperature equilibration, perform two complete buffer runs without injecting a sample.
9. Dilute the 250 µL sample in 375 µL of 1x Dilution Buffer to get a final volume of 625 µL (1 to 2.5 dilution).
10. Remove any sample particulates using a 0.45 µm spin filter. Centrifuge for 1 minute at 9200 x *g*.
11. Inject 625 µL of the diluted sample and start the separation method.
12. Collect flow-through and eluted fractions. Add 1/10 volume of 10x Neutralization Buffer (1.6-1.8 mL) to eluted fraction (16-18 mL). Store both fractions at -70°C if they are not analyzed immediately.
13. If the column is not going to be reused within 7 days, equilibrate the column with 1x Dilution Buffer that contains 0.02% (w/v) sodium azide. Start the 32 Karat software and select `IgYLC10_store.met` method file on the ProteomeLab PPS System, or pump the 1x Dilution Buffer with 0.02% sodium azide at a flow rate of 1.0 mL/min. for 20 min. through the column on other chromatographic systems. Store the column at 2-8°C in a refrigerator.

    DO NOT FREEZE THE COLUMN.

14. Concentrate the flow through and bound protein samples with the Amicon Ultra-15 centrifugal filter unit, 5,000 molecular weight limit according to the protein concentration instructions as provided by the manufacturer.

NOTE: For further fractionation using the ProteomeLab PF 2D, continue with the next 2 steps.

15. After concentrating the flow-through and/or the stripped protein sample to a minimum volume, add ProteomeLab PF 2D Start Buffer to a final volume of 4.0 mL and concentrate again.
16. Finally, add a volume of Start Buffer to bring the final volume to 2.0 mL for injection on the ProteomeLab PF 2D.

It is suggested that 1-5 mg of protein be injected on the ProteomeLab PF 2D, which will require 1 to 5 cycles of the LC10 column.

Expected Performance

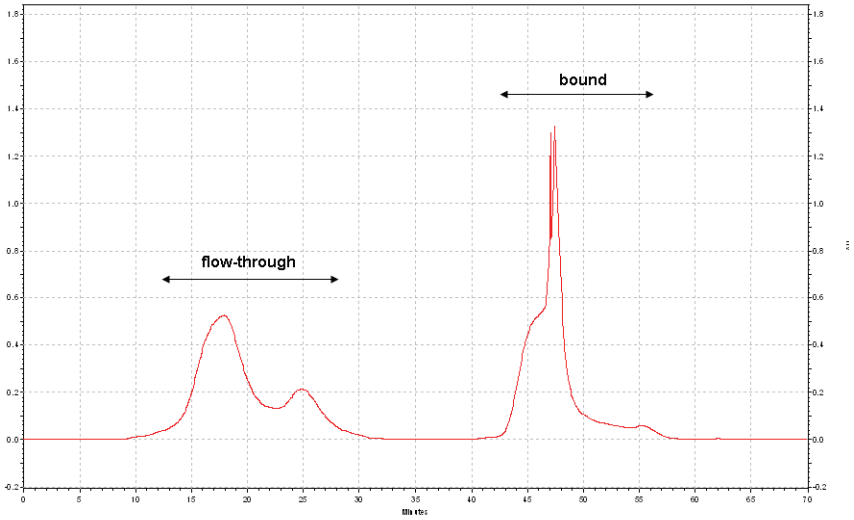


Figure 4: Chromatography of Human Plasma/ Serum using ProteomeLab IgY-12 LC10. 125 µL of human plasma was partitioned on the column. Note that the A_{280} absorbance of the flow-through peak appears to be less than the bound peak. The expected volume of the flow-through fractions is 9-11 mL. The expected volume of the bound fractions is 16-18 mL.

Troubleshooting

Symptom	Possible Cause	Corrective Action
Reduction in protein binding with abnormal capacity peak height	• Column may have not been regenerated well enough from previous runs.	• Elute bound proteins with Stripping Buffer for 2 additional column volumes, and then immediately neutralize and re-equilibrate the column with 1x Neutralization Buffer and Dilution Buffer.
	• Buffers contaminated with bacteria or fungi.	• Carefully check for signs of biological growth and precipitates in the Buffers. Replace with fresh Buffers for optimized column performance.
High Backpressure or Distorted peak shape	Clogged inlet frits	Remove particulates from all samples with a 0.45 µm spin filter before loading. Failure to pre-filter the sample can diminish the column lifetime.
No bound fraction peak	• Insufficient elution with Stripping Buffer. Bound proteins should only be removed from the column using Stripping Buffer.	• Check LC timetable to ensure enough column time exposure to Stripping Buffer for complete removal of bound proteins.
	• Column may not have been regenerated well enough from previous runs, resulting in lost capacity.	• To correct this, elute bound proteins with Stripping Buffer for 2 additional column volumes, and then neutralize and re-equilibrate the column with Neutralizing Buffer and Dilution Buffer.
	• Buffers contaminated with bacteria or fungi.	• Check for signs of biological growth in the reservoirs. Replace with fresh buffers for optimized column performance.

For Beckman Coulter contact information, see “Additional Technical Support” on page 8.

Recommendations

- **Sample dilution**
  It is not recommended to load crude serum directly onto the column. Follow instructions for plasma or serum dilution (five times dilution with Dilution Buffer).
- **Column performance**
  Adjust LC timetable based on your own instrument if necessary. Do not expose the columns to solvents other than the three provided buffers (Dilution, Stripping, and Neutralization).
- **Column storage**
  Always store the columns after equilibrating with Dilution Buffer in a refrigerator at 2-8°C when not in use to minimize loss in column capacity. Dilution Buffer should contain 0.02% (w/v) sodium azide for prolonged column storage greater than 7 days.
- **Lyophilization of flow-through fractions**
  Buffer exchange to a volatile buffer (for example, ammonium bicarbonate) is recommended prior to lyophilization due to high salt concentration in Dilution Buffer.

Caution: Do not expose columns to organic solvents (like alcohols, acetonitrile, etc.), strong oxidizers, acids, or reducing agents, and other protein denaturing agents.

Warning: FOR RESEARCH USE ONLY - this product is NOT TO BE USED AS AN *IN-VITRO* DIAGNOSTIC.

Storage: Store the affinity column at 2-8°C upon receiving and when not in use. DO NOT FREEZE THE COLUMN!

Appendix A: IgY-12 High Capacity LC2

Table 1: LC Method for 6.4 x 63 mm LC2 Column

The table below provides a starting point for developing a method file for use with the LC2 Column. The detector wavelength should be set to 280 nm and 1 Hz data collection rate.

Cycle	Time (min)	Dilution Buffer	Stripping Buffer	Neutralization Buffer	Flow rate (mL/min)
Injection					
Wash	0	100	0	0	0.1
Wash	10.01	100	0	0	0.2
Wash	17.01	100	0	0	1.0
Stripping	22.01	0	100	0	1.0
Neutralization	36.01	0	0	100	1.0
Re-equilibration	42.01	100	0	0	1.0
Stop	48.00				

Appendix B: IgY-12 High Capacity LC10

Table 2: LC Method for 12.7 × 79.0 mm LC10 Column

The table below provides a starting point for developing a method file for use with non-Beckman Coulter LC Systems. The detector wavelength should be set to 280 nm and 1 Hz data collection rate.

Cycle	Time (min)	Dilution Buffer	Stripping Buffer	Neutralization Buffer	Flow rate (mL/min)
Injection					
Wash	0	100	0	0	0.5
Wash	25.01	100	0	0	2.0
Stripping	30.01	0	100	0	2.0
Neutralization	48.01	0	0	100	2.0
Re-equilibration	56.01	100	0	0	2.0
Stop	65.00				

Background Literature

1. **Adkins, J.N., S.M. Varnum, K.J. Auberry, R.J. Moore, N.H. Angell, R.D. Smith, D.L. Springer, and J.G. Pounds**. 2002. Toward a human blood serum proteome: analysis by multidimensional separation coupled with mass spectrometry. Mol Cell Proteomics *1*: 947-955.
2. **Anderson, N.L. and N.G. Anderson.** 2002. The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics *1*: 845-867.
3. **Anderson, N.L., M. Polanski, R. Pieper, T. Gatlin, R.S. Tirumalai, T.P. Conrads, T.D. Veenstra, J.N. Adkins, J.G. Pounds, R. Fagan, and A. Lobley.** 2004. The Human Plasma Proteome A Nonredundant List Developed by Combination of Four Separate Sources. Mol Cell Proteomics *3*: 311-326.
4. **Burgess-Cassler, A., J.J. Johansen, and N. Kendrick.** 1989. Immunodepletion of albumin from human serum samples. Clin Chim Acta *31*: 359-365.
5. **Burtis, C.A. and E.R. Ashwood.** 2001. Tietz Fundamentals of Clinical Chemistry. 5th Ed., W.B. Saunders Company, Philadelphia, PA.
6. **Corthals, G.L., V.C. Wasinger, D.F. Hochstrasser, and J.C. Sanchez.** 2000. The dynamic range of protein expression: a challenge for proteomic research. Electrophoresis *21*: 1104-1115.
7. **Davidsson, P., S. Folkesson, M. Christiansson, M. Lindbjer, B. Dellheden, K. Blennow, and A. Westman-Brinkmalm.** 2002. Identification of proteins in human cerebrospinal fluid using liquid-phase isoelectric focusing as a prefractionation step followed by two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionisation mass spectrometry. Rapid Commun Mass Spectrom *16*: 083-2088.
8. **Fang, X., K.W. Curran, L. Huang, W. Xiao, W. Strauss, G. Harvie, J. Feitelson, and W.-W. Zhang.** 2003. Polyclonal Gene-Specific IgY Antibodies for Proteomics and Abundant Plasma Protein Depletion, p. 222-245. In J. Reiner, K. Zhao, S.-H. Chen, and M. Guo, (Eds.), Frontiers of Biotechnology and Pharmaceuticals, Vol. 4, Science Press USA, Inc. Monmouth Junction, NJ.
9. **Fang, X., L. Huang, J. Feitelson, and W.-W. Zhang.** 2004. Affinity Separation: Divide and Conquer the Proteome. Drug Discovery Today: Technologies *1*: 141-148.
10. **Hadge, D. and H. Ambrosius.** 1984. Evolution of low molecular weight immunoglobulins - IV. IgY-like immunoglobulins of birds, reptiles and amphibians, precursors of mammalian IgA. Mol Immun *21*: 699-707.
11. **Heilmann, S.M., Rasmussen, J.K., Krepski, L.R., Milbrath, D.S., and P.L. Coleman.** 1989. Variably crosslinked polymeric supports. U.S. Patent 4,871,824.
12. **Huang, L., G. Harvie, J. S. Feitelson, K. Gramatikoff, D.A. Herold, D.L. Allen, R. Amunagama, R.A. Hagler, M.R. Pisano, W.-W. Zhang, and X. Fang.** 2005. Immunoaffinity Separation of Plasma Proteins by IgY Microbeads: Meeting the Needs of Proteomic Sample Preparation and Analysis. *Proteomics 5*: 3314-3328.
13. **Kojima, K.** 2001. High-performance immunoaffinity chromatography, an immunoaffinity membrane for selective removal of plasma components, and safety evaluation of the latter system. J Biochem Biophys Methods *49*: 241-251.
14. **Larsson, A., R.M. Balow, T.L. Lindahl, and P.O. Forsberg.** 1993. Chicken antibodies: Taking advantage of evolution - A review. Poultry Science *72*: 1807-1812.

15. **Larsson, A. and H. Mellerstedt.** 1992. Chicken antibodies: a tool to avoid interference by human anti-mouse antibodies in ELISA after *in vivo* treatment with murine monoclonal antibodies. Hybridoma *11*: 33-39.
16. **Lescuyer, P., D.F. Hochstrasser, and J.C. Sanchez.** 2004. Comprehensive proteome analysis by chromatographic protein prefractionation. Electrophoresis *25*:1125-1135.
17. **Leslie, G.A. and L.W. Clem.** 1969. Phylogeny of immunoglobulin structure and function. 3. Immunoglobulins of the chicken. J Exp Med *130*: 1337-1352.
18. **Lynch, M.** 2005. An Automated Method for Highly Abundant Protein Partitioning of Human Plasma Samples Prior to Proteomic Analysis. American Biotechnology Laboratory *23* (17): 24-26
19. **Pieper, R., C.L. Gatlin, A.J. Makusky, P.S. Russo, C.R. Schatz, S.S. Miller, Q. Su, A.M. McGrath, M.A. Estock, P.P. Parmar, M. Zhao, S.T. Huang, J. Zhou, F. Wang, R. Esquer-Blasco, N.L. Anderson, J. Taylor, and S. Steiner.** 2003. The human serum proteome: display of nearly 3700 chromatographically separated protein spots on two-dimensional electrophoresis gels and identification of 325 distinct proteins. Proteomics *3*:1345-1364.
20. **Pieper, R., Q. Su, C.L. Gatlin, S.T. Huang, N.L. Anderson, and S. Steiner.** 2003. Multi-component immunoaffinity subtraction chromatography: an innovative step towards a comprehensive survey of the human plasma proteome. Proteomics *3*: 422-432.
21. **Rothemund, D.L., V.L.Locke, A.Liew, T.M.Thomas, V.Wasinger, and D.B. Rylatt.** 2003. Depletion of the highly abundant protein albumin from human plasma using the Gradiflow. Proteomics *3*: 279-87.
22. **Schade, R. and A. Hlinak.** 1996. Egg Yolk Antibodies, State of the Art and Future Prospects. ALTEX *13*: 5-9.
23. **Schneider, I.** 2004. Improving productivity in protein research. Gen Eng News 24: 21-25.
24. **Tam, S.W., J.L. Pirro, and D. Hinerfeld.** 2004. Depletion and Fractionation Technologies in Plasma Proteomic Analysis. Expert Rev. Proteomics 1: 411-20.
25. **Tam, S.W., Huang, L., Hinerfeld, D., Innamorati, D., Fang, X., Zhang, W.-W., Pirro, J. & Feitelson, J.S.** 2005. Novel plasma protein separation startegy using mulitple avian IgY antibodies for proteomic analysis. In: Separation Methods in Proteomics (Smejkal, G.B. & Lazarev, A. eds.) Marcel Dekker.
26. **Vesterberg, O., and H. Anundi.** 1991. Identification of human blood plasma and serum proteins in two-dimensional gels by use of protein A-Sepharose: application to alpha 1-microglobulin. Appl Theor Electrophor *2*:159-161.
27. **Warr, G.W., K.E. Magor, and D.A. Higgins.** 1995. IgY: clues to the origins of modern antibodies. Immunol Today 16: 392-398.
28. **Zhang, W.-W.** 2003. The Use of Gene-Specific IgY Antibodies for Drug Target Discovery. Drug Discovery Today *8*: 364-371.
29. **Zheng, L., Nukuna, B., Brennan, M.L., M. Sun, M. Goormastic, M. Settle, D. Schmitt, X. Fu, L. Thomson, P.L. Fox, H. Ischiropoulos, J.D. Smith, M. Kinter, S.L. Hazen.** 2004. Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. J Clin Invest. 114, 529-541.