New Proteomic Subfractionation Surfaces – Innovative Technology For The Improved Resolution of Serum Proteins

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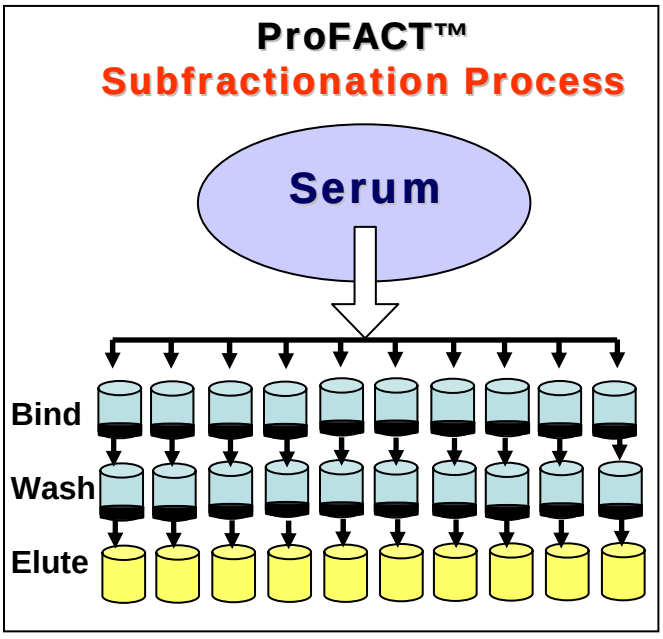
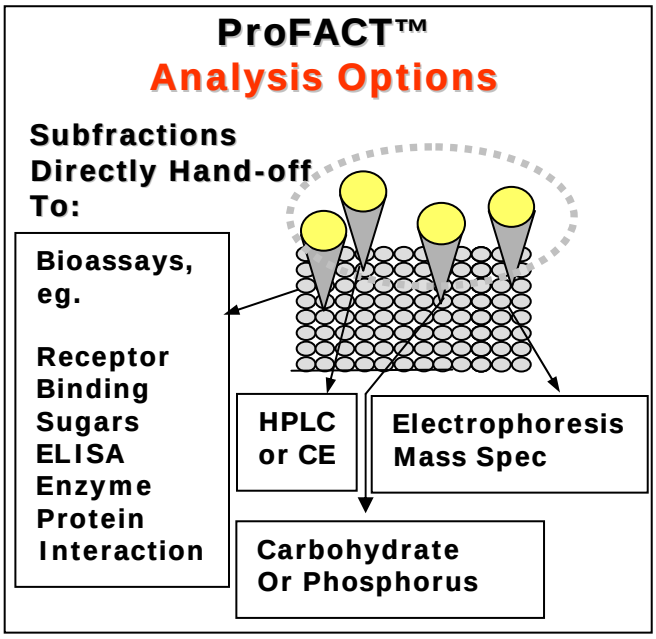




Introduction

ProFACT™ is a new subfractionation methodology designed for comparative proteome analysis. Electrophoretic profiles of serum subfractions demonstrate improved resolution and quantification. Carryover from the three highest abundance serum proteins, Albumin, IgG and Transferrin is minimal. The process starts with a separation platform utilizing a new combination of surface microenvironments substituted with low molecular weight substrates that feature drug-binding motifs. With the **ProFACT™** surface library, **undenatured, bioactive** proteins can be subfractionated into differential pools. Separations are universal as they do not require pre-qualified binding knowledge, a key limitation of affinity-type techniques. The surfaces utilized are disposable and adaptable to sample size and scale requirements. A simple bind, wash and elute protocol is completed in 30 to 60 minutes and as elutions are mild and consistent, a direct handoff can be made to subsequent interrogation. The interrogation strategy is adaptable to meet investigative inquiry using 'bottom-up' or 'top-down' approaches.

Detailed iterative profiling can be applied towards biomarker discovery, disease-state pattern identification, systems biology, or otherwise be useful to reduce sample complexity. The ProFACT™ surface library can potentially be coupled with HPLC, Capillary Electrophoresis, 1 and 2D Electrophoresis, and Mass Spectrometry to expand coverage and sensitivity. As structural modifications of proteins can alter their binding affinities to the surface library, structural differences in sample sets may be inferred upon interpretation of ProFACT™ subfraction profiles.

The data presented herein demonstrate the unique profiling capabilities of each ProFACT™ surface library subfraction and the collective resolution of 69 non-redundant proteins, calibrated and quantified from image analysis of SDS-PAGE profiles. 10 differential protein subfractions can be generated in less than 1 hour, without the need for immuno-depletion. Future investigations will focus on comparing normal and disease state sera.

Background and Significance

Proteomics is encumbered by complexity, unreliable quantification, low-throughput technologies, and lacks systemic ability to uncover structural and functional isoform changes. Recent attention has attempted to reduce the complexity of protein samples, particularly with serum due to the presence of three major protein regions: Albumin, Transferrin and Immunoglobulin. To address this problem, several products have been introduced that have selective binding properties towards one or more of the high abundance proteins in serum. These work through high affinity interactions, most notably using immuno-affinity. Subsequent resolution techniques typically include either 2-dimensional electrophoresis (2DE) or multi-dimensional HPLC. While productive, these methods generally are:

- costly for large sample sets,
- cumbersome and low throughput,
- at best only moderately quantifiable,
- generate limited structural information,
- not providing intact, bioactive protein pools.

ProFACT™ is a new subfractionation surface library intended to address these fundamental problems.

Complexity Reduction Strategy

The ProFACT™ process begins by first subfractionating serum, then analyzing, comparing and contrasting each subfraction individually. 1-dimensional electrophoresis is well-suited for this task as it is quick, reproducible and offers precise peak resolution capability and relative peak calibration and quantification after analysis through commercially available image software.

From each ProFACT™ surface subfraction, both an electropherogram and digital profile is generated. The strategy in principal is to identify differences in one or more subfractions through comparative analysis between samples. Differences in subfractions are the "hit" subfractions and only these need to be analyzed further. These "hit" subfractions contain a reduced protein complexity and proteins are pooled with structural and functional integrity intact, potentially valuable in secondary bioassays.

Figure 1

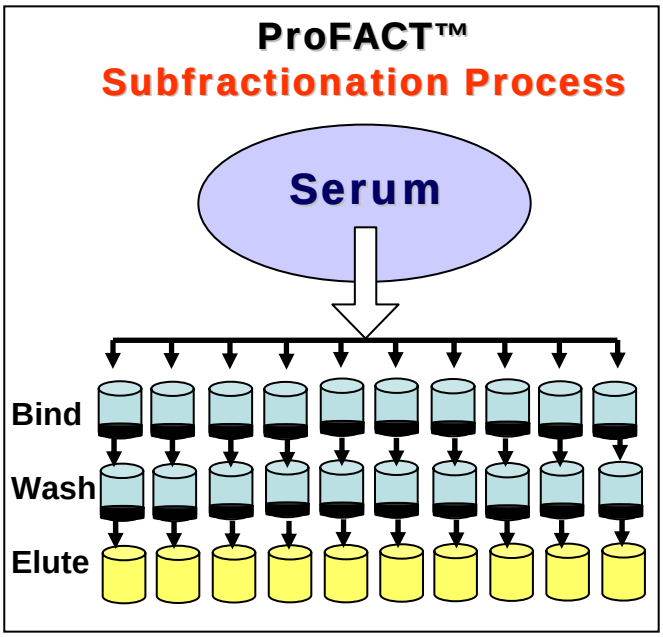
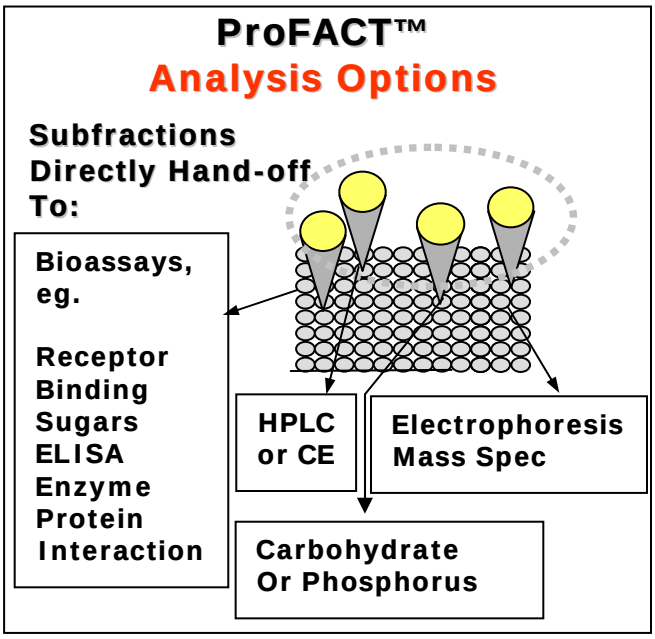


Figure 2



ProFACT™ Subfractionation Surface Library

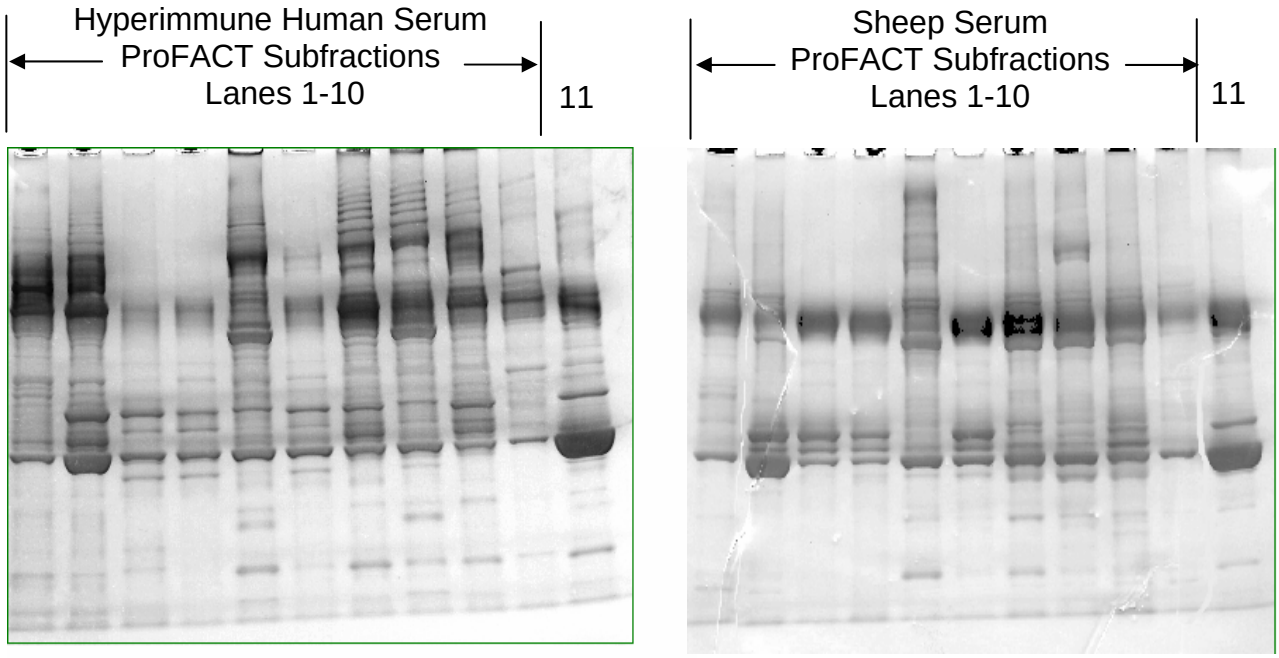
The ProFACT™ surface library is constructed by spatially presenting drug-binding motifs within microenvironments such that binding interactions occur homogeneously with proteins. Unlike conventional chromatography, **ProFACT™ surfaces are not subject to the predominant influence of protein concentration**. Therefore high abundance proteins compete with low abundance proteins for each surface selectivity site. **The net result is a substantial voiding of the high abundance proteins and subsequent unmasking of the low abundance proteins**. Thus, the surface library produces distinctive protein pools that can be immediately analyzed by high resolution electrophoretic and potentially other bioarray techniques. In the analysis that follows, 10 ProFACT™ subfractionating surfaces are shown to modulate selectivity on a pooled human serum sample. Simple bind (pH 6), wash and elute (pH 9) protocols (Figure 1) provide a reduction of complexity, each differentiated pool ready for subsequent analysis (Figure 2).

Experiment Design

In order to utilize the proposed complexity reduction strategy, we needed to first investigate the utility of combining the ProFACT™ surface library with 1-dimensional SDS-PAGE. By demonstrating the differentiation of each subfraction, new proteomic profile capabilities and strategies become available.

ProFACT™ surfaces are constructed from silica specially adapted to meet the requisite separation characteristics. Each surface was weighed (0.25 Grams) and placed into spin microtubes such that the total volume of wet surface was approximately 50 µls. Each surface was washed with pH 6 binding buffer, twice using 400 µls. 0.5 ml of serum was diluted to 5 ml with pH 6 binding buffer and 300 µls of the diluted serum applied to each surface. All surfaces were then shaken for 10 minutes and centrifuged, the surfaces washed 2X with pH 6 buffer, and then eluted using 100 µls of a pH 9 buffer. The total separation time was about 1 hour. ODs were taken with the Nanodrop® spectrophotometer. Samples were dried and then resuspended in 10 µls Tris-Glycine-SDS sample buffer. Each subfraction was applied to the gel for electrophoresis. Non-reducing electrophoresis conditions were: 4-20% precast gel (Invitrogen), 130 volts, run for 90 minutes and stained with Simple Blue Stain™ (Invitrogen).

SDS-PAGE Profile of ProFACT™ Subfractionated Human and Sheep Serums



Lane 11 – Serum Controlsl Untreated

Results and Discussion

After de-staining, the gel was scanned and then analyzed with the image software TotalLab™ (Non-Linear Dynamics). Lanes 1-10 correspond with ProFACT™ surfaces 1-10; lane 11 is a serum control. We allow that there is subjectivity in the selection of peak picking parameters, and we have attempted to be moderate in their selection so as to have a reasonable estimate of the total number of resolved peaks in each lane and the aggregate number from all lanes combined. By manually adjusting the image boxes in each lane, distortions in the gel are compensated, Figure 3.

Each lane can then be scaled equivalently from 0 to 1, called the Retardation Factor, or “Rf”. Protein peaks can then be identified through their Rf number, i.e., all Rf proteins corresponding to 0.19 are assumed to be resolved the same and for discussion purposes are considered individual. With similar methods, overlays can illustrate the substantial reduction of high abundance peaks and the subsequent resolution of many underlying proteins from regions associated with Albumin, Transferrin, and Immunuglobulin.

Each individual lane presented below illustrates the derivative electropherogram profile and its associated lane image, Figure 4. An overlay of each lane compared to the serum lane control, is encompassed in Figure 5.

Figure 3
TotalLab™ Image Analysis of Each Lane

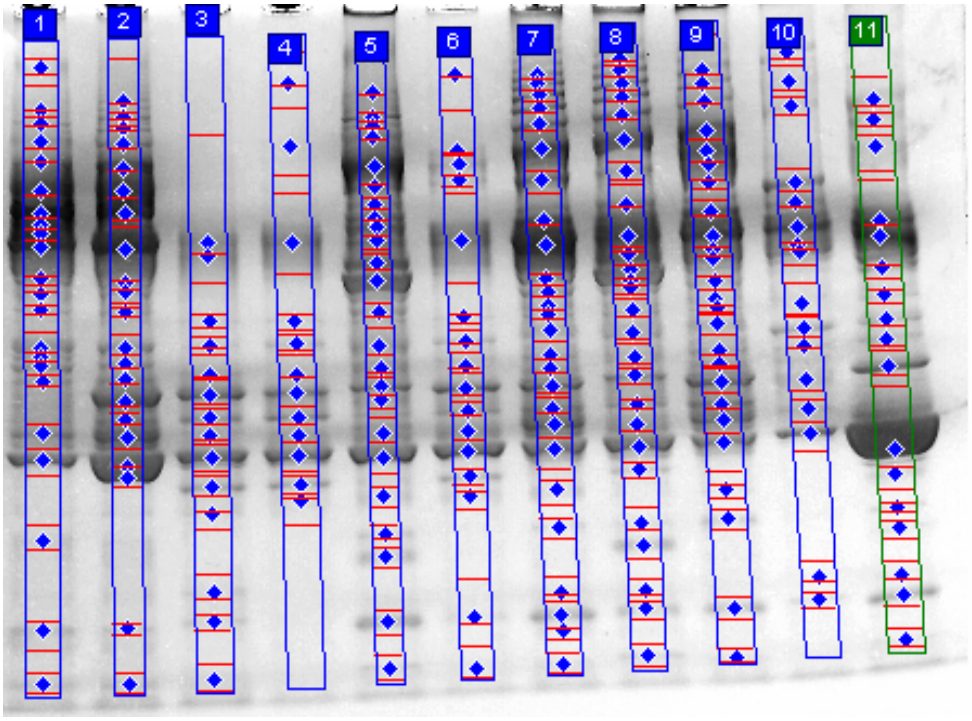
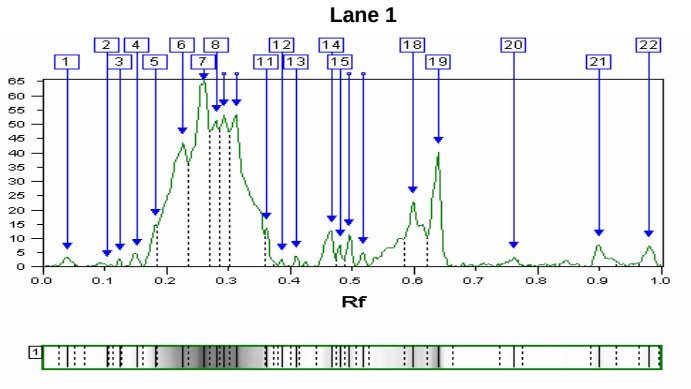
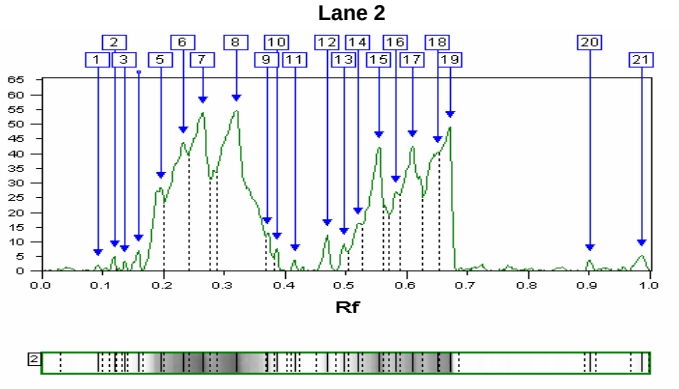


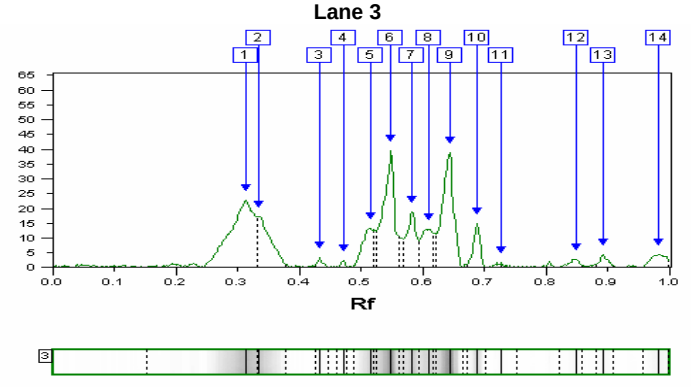
Figure 4
Lane by Lane Analysis of Each Boxed Image and Its Derivative Electropherogram



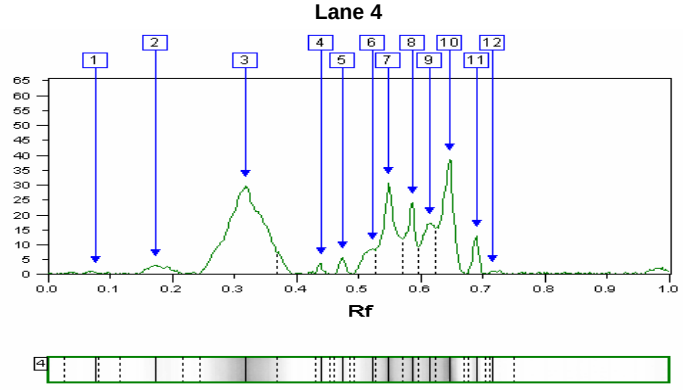
Total Resolved Peaks in Lane = 22
*Non-Redundant Peaks in Lane = 9



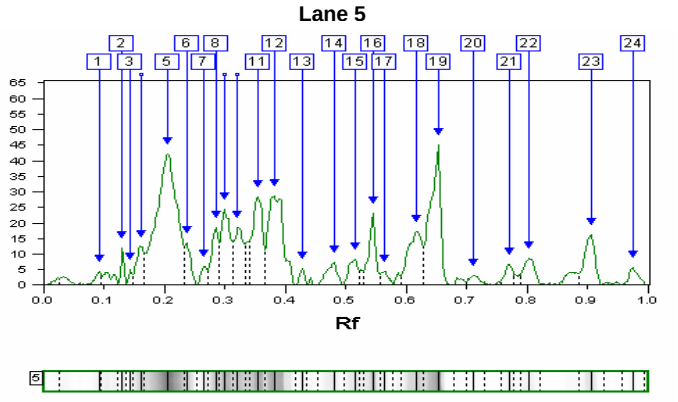
Total Resolved Peaks in Lane = 21
*Non-Redundant Peaks in Lane = 6



Total Resolved Peaks in Lane = 14
*Non-Redundant Peaks in Lane = 3



Total Resolved Peaks in Lane = 10
*Non-Redundant Peaks in Lane = 0

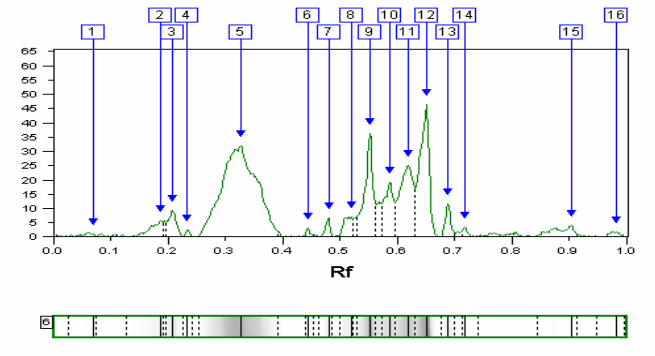


Total Resolved Peaks in Lane = 26
*Non-Redundant Peaks in Lane = 4

*Non-Redundant Peaks is a measure of the peaks that are resolved only in this subfraction or, only in this subfraction and one other subfraction. Data was taken from the Excel Spreadsheet analysis that follows.

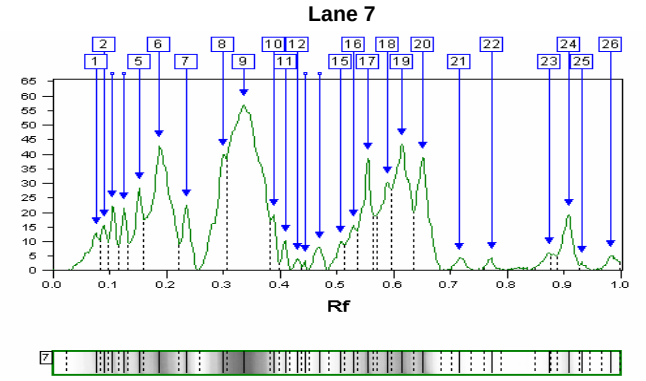
Figure 4 (Continued)
Lane by Lane Analysis of Each Boxed Image and Its Derivative Electropherogram

Lane 6



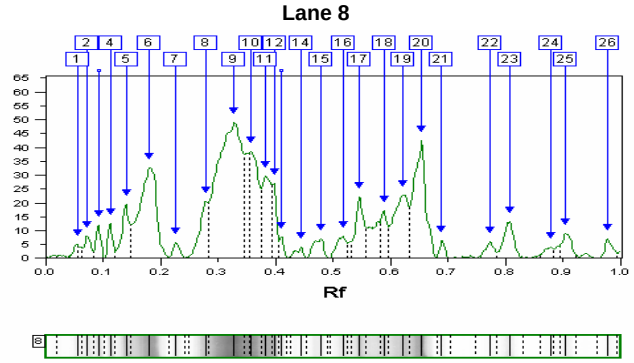
Total Resolved Peaks in Lane = 15
*Non-Redundant Peaks in Lane = 2

Lane 7



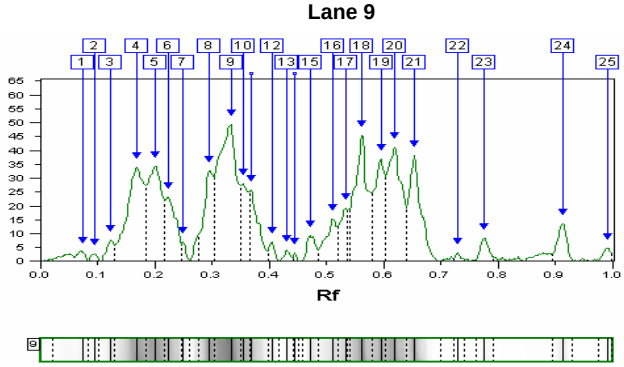
Total Resolved Peaks in Lane = 25
*Non-Redundant Peaks in Lane = 7

Lane 8



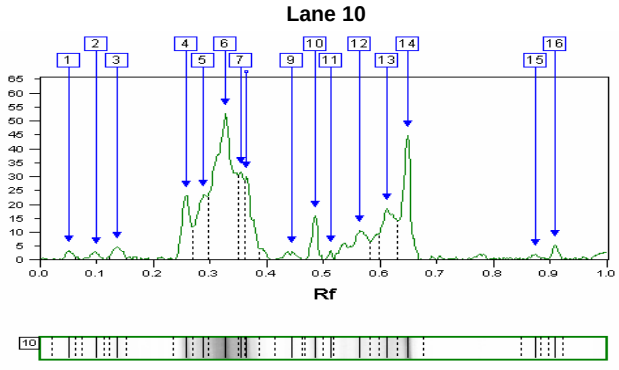
Total Resolved Peaks in Lane = 26
*Non-Redundant Peaks in Lane = 6

Lane 9



Total Resolved Peaks in Lane = 25
*Non-Redundant Peaks in Lane = 10

Lane 10

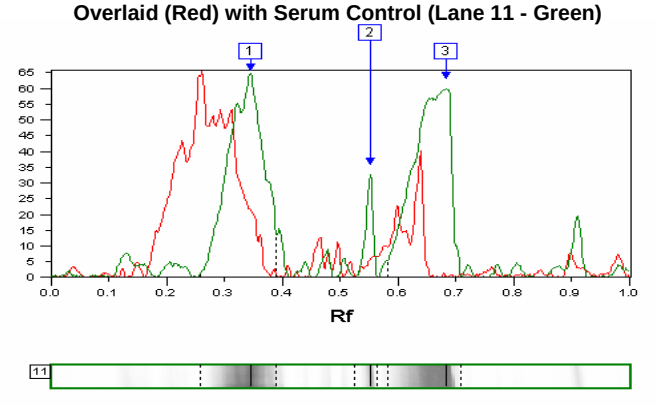


Total Resolved Peaks in Lane = 16
*Non-Redundant Peaks in Lane = 3

*Non-Redundant Peaks is a measure of the peaks that are resolved only in this subfraction or, only in this subfraction and one other subfraction. Data was taken from the Excel Spreadsheet analysis that follows.

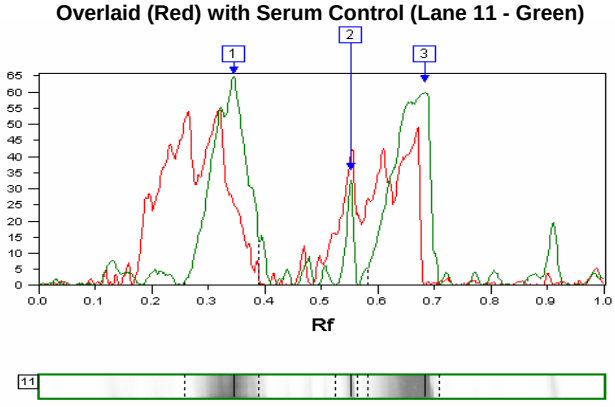
Figure 5
Lane by Lane Analysis vs. Serum Control

Lane 1
Overlaid (Red) with Serum Control (Lane 11 - Green)



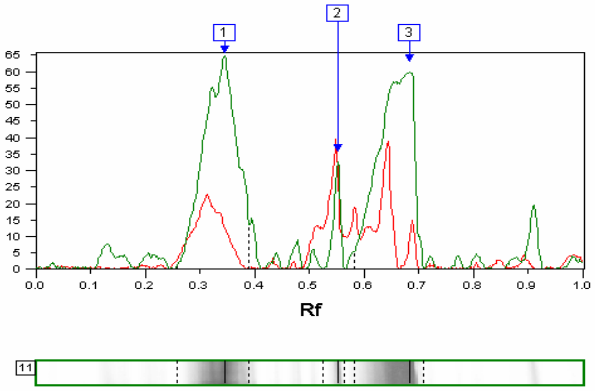
1 = Immunoglobulin Region Unmasked
2 = Transferrin Region Unmasked
3 = Albumin Region Unmasked

Lane 2
Overlaid (Red) with Serum Control (Lane 11 - Green)



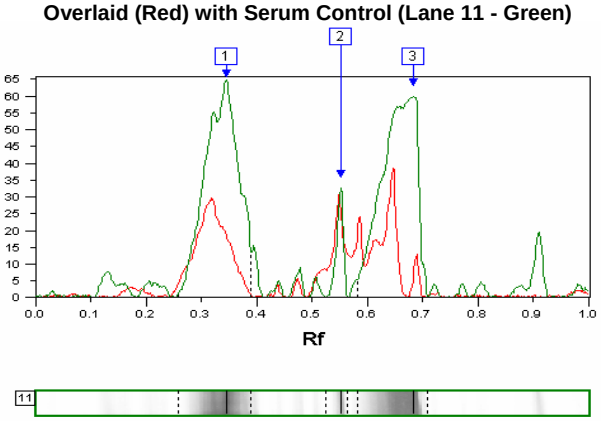
1 = Immunoglobulin Region Unmasked
2 = Transferrin Region
3 = Albumin Region Unmasked

Lane 3
Overlaid (Red) with Serum Control (Lane 11 - Green)



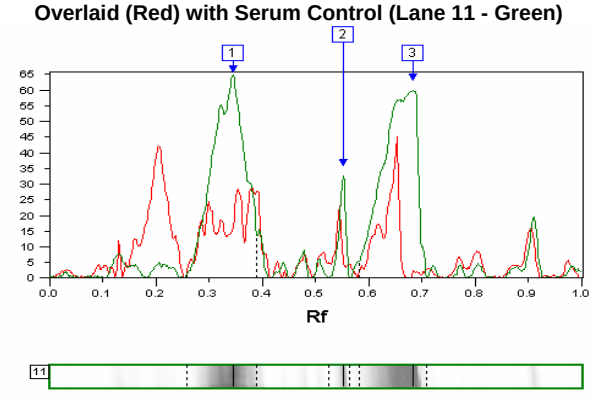
1 = Immunoglobulin Region
2 = Transferrin Region
3 = Albumin Region Unmasked

Lane 4
Overlaid (Red) with Serum Control (Lane 11 - Green)



1 = Immunoglobulin Region
2 = Transferrin Region
3 = Albumin Region Unmasked

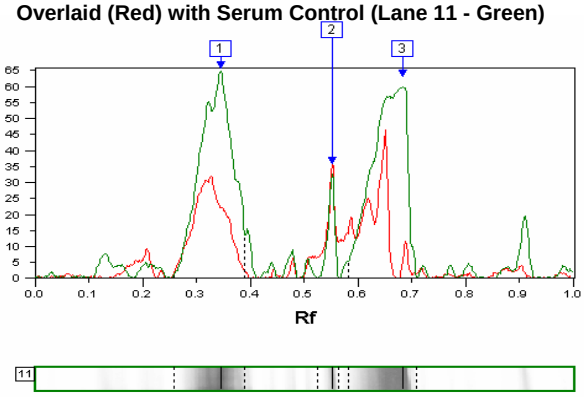
Lane 5
Overlaid (Red) with Serum Control (Lane 11 - Green)



1 = Immunoglobulin Region Unmasked
2 = Transferrin Region
3 = Albumin Region Unmasked

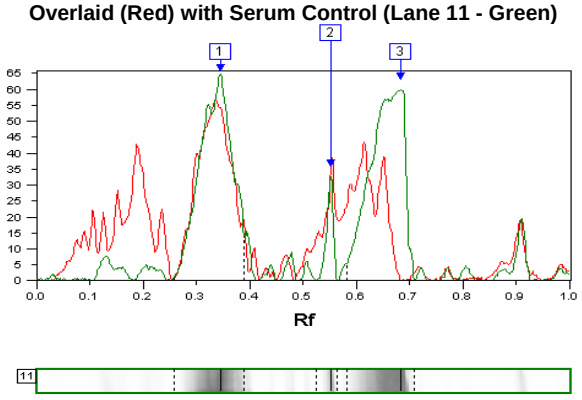
Figure 5 (Continued)
Lane by Lane Analysis vs. Serum Control

Lane 6
Overlaid (Red) with Serum Control (Lane 11 - Green)



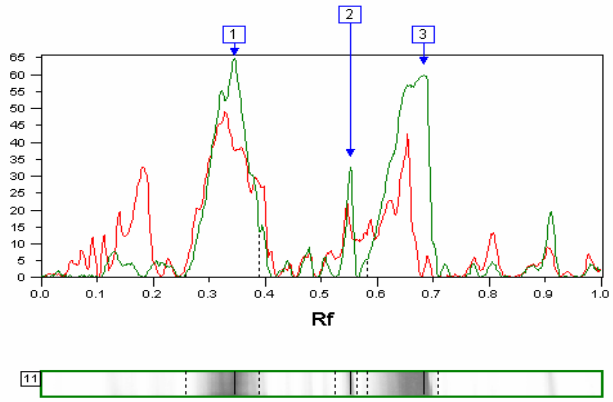
1 = Immunoglobulin Region
2 = Transferrin Region
3 = Albumin Region Unmasked

Lane 7
Overlaid (Red) with Serum Control (Lane 11 - Green)



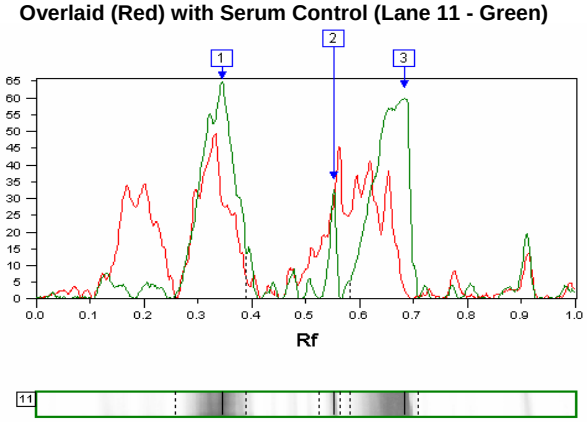
1 = Immunoglobulin Region
2 = Transferrin Region
3 = Albumin Region Unmasked

Lane 8
Overlaid (Red) with Serum Control (Lane 11 - Green)



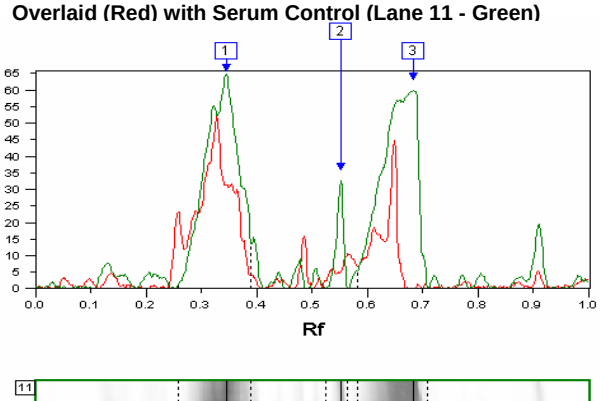
1 = Immunoglobulin Region
2 = Transferrin Region
3 = Albumin Region Unmasked

Lane 9
Overlaid (Red) with Serum Control (Lane 11 - Green)



1 = Immunoglobulin Region
2 = Transferrin Region Unmasked
3 = Albumin Region Unmasked

Lane 10
Overlaid (Red) with Serum Control (Lane 11 - Green)



1 = Immunoglobulin Region Unmasked
2 = Transferrin Region Unmasked
3 = Albumin Region Unmasked

Excel Data Analysis

Band. Band numbers correspond to the gel image peak analysis.

Volume. TotalLab™ software integrates all the pixels from each resolved protein peak and quantifies each as volume data. Volume data is relative and may be useful in algorithms when comparing peaks from one lane to the next or when measuring sample to sample variance. Peak height to volume data algorithms may reveal structural isoforms, especially glycosylation which tends to spread bands.

Rf. The Retardation Factor or Rf, is a scale from 0 to 1, that allows the software to compensate for lane to lane gel distortions, enabling a precise calibration when comparing lanes. Therefore, all peaks possessing the same Rf with a 1% threshold variance, are characterized as equivalent in this analysis.

By exporting the TotalLab™ data to an Excel spreadsheet, all of the Rf equivalent peaks can be aligned and interpreted.

Results and Interpretation:

- Collectively, 200 individual peaks are resolved,
- There are 69 Rf equivalents with a 1% threshold variance,
- Each subfraction lane provides a unique profile,
- Individual peaks are quantified and calibrated,
- Profiles contain a range of redundant, semi-redundant and non-redundant peaks,
- Unique subfractions based upon structural features, not possible with alternative techniques.

Progressive Peak Number	Peak Redundancy (# of Lanes)	Lane 1			Lane 2			Lane 3			Lane 4			Lane 5			Lane 6			Lane 7			Lane 8			Lane 9			Lane 10		
		Band	Volume	Rf	Band	Volume	Rf	Band	Volume	Rf	Band	Volume	Rf	Band	Volume	Rf	Band	Volume	Rf	Band	Volume	Rf	Band	Volume	Rf	Band	Volume	Rf	Band	Volume	Rf
1	4	1	52949	0.05										1	41261	0.04							1	51279	0.05				1	45799	0.05
2	5										1	60040	0.07				1	55583	0.07	1	90291	0.07	2	28259	0.07	1	97684	0.07			
3	4													2	35184	0.09				2	26536	0.09	3	32124	0.09	2	37631	0.09			
4	3				1	122933	0.10													3	30486	0.10							2	48051	0.10
5	2	2	57068	0.11																			4	32466	0.11						
6	2																			4	33495	0.12				3	39457	0.12			
7	3	3	22433	0.13	2	21071	0.13							3	17127	0.13															
8	4				3	15310	0.14							4	14367	0.14							5	49003	0.14				3	46233	0.14
9	1																			5	47659	0.15									
10	2	4	60020	0.16										5	28058	0.16															
11	3				4	48928	0.17	1	194772	0.17																4	114419	0.17			
12	1																						6	123205	0.18						
13	2	5	42728	0.19																6	126198	0.19									
14	2				5	79530	0.20																			5	73638	0.20			
15	2													6	115771	0.21	2	123481	0.21												
16	8	6	119938	0.24	6	99410	0.24							7	31086	0.24	3	20980	0.23	7	62602	0.23	7	43940	0.23	6	56190	0.22	4	48419	0.22
17	6	7	92760	0.27	7	96109	0.27							8	27311	0.27							8	54968	0.28	7	24842	0.25	5	63549	0.26
18	4	8	40866	0.29										9	26656	0.29										8	54202	0.29	6	47101	0.29
19	3	9	45526	0.30										10	38643	0.30				8	92363	0.30									
20	5	10	129899	0.32	8	196948	0.33	2	135566	0.32	2	223218	0.32	11	26633	0.32															
21	4																4	213373	0.33				9	133655	0.33	9	108839	0.33	7	109399	0.33
22	2							3	78551	0.34										9	179472	0.34									
23	3													12	38358	0.35										10	32105	0.35	8	21743	0.35
24	1																						10	42853	0.36						
25	3	11	25206	0.37																						11	55791	0.37	9	47269	0.37
26	3				9	22384	0.38							13	76406	0.38							11	36989	0.38						
27	3	12	33883	0.39	10	32582	0.39													10	26989	0.39									
28	1																						12	21941	0.40						
29	3																			11	30005	0.41	13	21290	0.41	12	29929	0.41			
30	2	13	22309	0.42	11	22855	0.42																								
31	3													14	23465	0.43				12	30978	0.43				13	36654	0.43			
32	5							4	30081	0.44	3	17319	0.44				5	16993	0.44	13	17694	0.44	14	38198	0.44						
33	2																									14	15561	0.45	10	68395	0.45
34	6	14	54444	0.47	12	47878	0.47	5	25284	0.47	4	31288	0.47							14	52065	0.47				15	45867	0.47			
35	4	15	24064	0.48										15	52025	0.48	6	30804	0.48				15	54343	0.48						
36	1																												11	49441	0.49
37	2	16	36763	0.50	13	33570	0.50																								
38	3																			15	42054	0.51				16	53046	0.51	12	26884	0.51
39	7	17	32929	0.52	14	45213	0.52	6	53177	0.52	5	47007	0.52	16	35752	0.52	7	33025	0.52				16	47083	0.52						
40	1																			16	36401	0.53									
41	1																									17	26484	0.54			
42	6							7	71502	0.55	6	68894	0.55	17	38124	0.55	8	50930	0.55	17	52991	0.55	17	39513	0.55						
43	3				15	94685	0.56							18	26416	0.56										18	73534	0.56			
44	1																												13	103760	0.57
45	1				16	34759	0.58																								
46	5							8	41920	0.59	7	39295	0.59				9	33371	0.59	18	43306	0.59	18	24920	0.59						
47	2	18	69720	0.60																						19	46366	0.60			
48	4				17	82319	0.61	9	41251	0.61	8	41586	0.61																14	55655	0.61
49	5													19	48757	0.62	10	55850	0.62	19	75501	0.62	19	56726	0.62	20	69652	0.62			
50	1	19	73653	0.64																											
51	7				18	43893	0.65	10	77592	0.65	9	72945	0.65	20	67008	0.65	11	68615	0.65	20	79078	0.65							15	65499	0.65
52	2																						20	80232	0.66	21	92033	0.66			
53	1				19	76801	0.67																								
54	4							11	53515	0.69	10	57342	0.69				12	30295	0.69				21	28745	0.69						
55	1													21	36805	0.71															
56	2																13	31598	0.72	21	44777	0.72									
57	1																									22	23941	0.73			
58	1	20	54427	0.76																											
59	3													22	22794	0.77				22	38865	0.77	22	43252	0.77						
60	1																									23	38213	0.78			
61	2													23	32063	0.81							23	49835	0.81						
62	1							12	53892	0.85																					
63	3													24	29299	0.88				23	33426	0.88	24	30530	0.88						
64	1							13	35457	0.89																					
65	2	21	63528	0.90	20	27342	0.90																								
66	5													25	42377	0.91	14	71251	0.91	24	52440	0.91	25	34249	0.91				16	83947	0.91
67	1																									24	46135	0.92			
68	6	22	45837	0.98	21	45709	0.98	14	57285	0.98				26	27920	0.98	15	53643	0.98				26	45406	0.98						
69	2																			25	29324	0.99				25	24108	0.99			

	Total Peaks	Lane 1=	22		Lane 2=	21		Lane 3=	14		Lane 4=	10		Lane 5=	26		Lane 6=	15		Lane 7=	25		Lane 8=	26		Lane 9=	25		Lane 10=	16	
# Peaks in	1 or 2 Lanes	Lane 1=	9		Lane 2=	6		Lane 3=	3		Lane 4=	0		Lane 5=	4		Lane 6=	2		Lane 7=	7		Lane 8=	6		Lane 9=	10		Lane 10=	3	

Future R&D

We intend to focus future R&D efforts so as to:

- establish quantitative standards for serum,
- use data for predictive insight in developing new ProFACT™ surfaces,
- develop ProFACT™ HPLC to enhance discovery and for top-down proteomics.

Conclusions

ProFACT™ offers new alternatives for comparison proteomic analysis offering many advantages.

- Unmask low abundance from high abundance,
- Immuno-depletion not required,
- Multi-subfractionation process < 1 hour,
- Adaptable to high throughput, multiwell systems,
- Intact protein analysis,
- Protein-Protein interaction,
- Focus electrophoretic parameters on MW regions of interest,
- Selection of protein subfraction 'hits' reduces complexity,
- Precise quantitative comparisons and algorithms,
- Structural isoforms may be inferred.