Clinical Proteomics Journal
Copyright ©Humana Press Inc.
All rights of any nature whatsoever are reserved.
ISSN 1542-6416/04/01:17–31/$25.00

Serum/Plasma Proteome

An Investigation of Plasma Collection, Stabilization, and Storage Procedures for Proteomic Analysis of Clinical Samples

Jeffrey D. Hulmes,[*,1] Deidra Bethea,[1] Keith Ho,[1] Shu-Pang Huang,[2] Deborah L. Ricci,[3] Gregory J. Opiteck[1] Stanley A. Hefta[1]*

[1]Department of Clinical Discovery, Proteomics Unit, Pharmaceutical Research Institute, Bristol-Myers Squibb Company, Princeton, NJ
[2,*]Department of Clinical Discovery, Biostatistics Unit, Pharmaceutical Research Institute, Bristol-Myers Squibb Company, Princeton, NJ
[3,*]Department of Clinical Discovery, Pharmacology Unit, Pharmaceutical Research Institute, Bristol-Myers Squibb Company, Princeton, NJ

Abstract

In order to evaluate the critical components of the process necessary to preserve clinical plasma samples collected at research sites for proteomic analysis, various collection and preservation protocols with controlled experimentation were evaluated. The presence of a protease inhibitor cocktail (PIC) included in the blood draw tube would stabilize the plasma proteins was hypothesized. To test this hypothesis, four plasma samples from each of 14 volunteers were collected. Samples were treated following a standard protocol that included PIC or were subjected to various processing treatments that included time, temperature, different anticoagulants, and the absence of PIC. Large format two dimensional-polyacrylamide gel electrophoresis (2D-PAGE) proteomic analysis and enzyme immunoassay (EIA) were used to detect differences between the treatment groups. A novel 2D-PAGE quality scoring method was developed to determine global differences in the treatment groups, wherein a rating scale questionnaire was used to quantify the quality of each 2D-PAGE gel. The data generated from EIAs, classical 2D-PAGE image analysis and 2D-PAGE quality scoring, each generated similar results. Inclusion of protease inhibitor cocktail in the sample tubes, provided stable and reliable human plasma samples that yielded reproducible results by proteomic analysis. When PIC was included, samples retained stability under less stringent processing, such that refrigeration for several hours before processing or one freeze–thaw cycle had little detrimental effect.

*Author to whom all correspondence and reprint requests should be addressed:
E-mail: jeffrey.hulmes@bms.com

We demonstrated that samples without PIC, from either heparin or ethylenediaminetetraacetic acid (EDTA) plasma tubes, gave results that varied significantly from the control samples. Also, even with PIC present in blood tubes, we found it was important to quickly decant the separated plasma from the cellular components found in the blood tubes following centrifugation, as prolonged exposure again yielded different results from the standard procedure.

Key Words: Proteomics; plasma collection; two-dimensional gel electrophoresis; sample stability; protease inhibitors; 2D-PAGE evaluation.

Introduction

Proteomic analysis is now being used extensively for the study of disease through the use of clinical samples such as tumor cell extracts, tissue samples, or body fluids *(1)*. Many of these studies involve the search for biomarkers in which certain proteins change in expression with either a large increase or decrease as a result of disease *(1–4)*. Two-dimensional gel electrophoresis (2-DE) remains the most widely used proteomic technique owing to the fact that it is currently the method that can separate and measure hundreds of proteins from a single biological sample simultaneously *(5,6)*. Analysis by 2D-PAGE has been used for proteomic studies of breast cancer *(7–9)*, prostate cancer *(10,11)*, hepatocellular carcinoma *(12,13)*, renal cell carcinoma *(14)*, rheumatoid arthritis *(15,16)*, and hepatitis B infection *(17)*. Many of these biomarker studies utilized human blood serum or plasma samples. The plasma proteome is now being widely studied and will be a rich source of proteins for proteomic analysis for years to come *(18)*.

The task of collecting large numbers of clinical plasma samples that best represent, and most accurately reflect, the plasma composition in vivo is of great importance. Given the large number of clinical trial sites around the world, it is important to standardize a procedure that will yield reliable samples for proteomic analysis. Clinical samples can be collected and processed in any number of ways, but can also be "mishandled." Plasma is often collected in a vacuum drawn blood tube that contains anticoagulants and a gel-separating barrier (e.g. VACUTAINER PPT™, Becton Dickinson, Franklin Lakes, NJ). There is a great possibility of lab-to-lab variations in sample handling and processing and it would be easy to identify, for instance, biomarkers of proteolysis rather than markers of a particular disease. The stability of some analytes, viral load, and a few proteins from collected plasma samples have been studied *(19–22)*, but to our knowledge the condition or stability of the whole plasma proteome from clinical samples has not been methodically examined. To minimize the possibility of generating "artifact" biomarkers, we have developed a very precise protocol for the collection of plasma samples from clinical trials. This protocol includes the addition of a protease inhibitor cocktail (PIC) directly to plasma collection tubes prior to phlebotomy. Marshall et al. have recently shown that phenylmethylsulfonyl fluoride (PMSF) and ethylenediaminetetraacetic acid (EDTA) effects the stability of serum samples as judged by matrix assisited laser desorption/ionization mass spectrometry (MALDI-MS) *(23)* while Olivieri et al. have previously demonstrated protease inhibitors in red blood cell membrane lysates can have large effect on 2D-PAGE analysis *(24)*. Here we present our findings of controlled treatments of plasma measured by proteomic analysis.

While it is logical that both a rigorous collection protocol and inclusion of PIC were necessary to obtain high quality plasma samples for proteomics analysis, neither had been thor-

Subject	A	B	C	D	E	F	G	H
1	X	X	X	X				
2	X	X	X	X				
3	X		X	X	X			
4	X		X	X	X			
5	X			X	X	X		
6	X			X	X	X		
7	X				X	X	X	
8	X				X	X	X	
9	X					X	X	X
10	X					X	X	X
11	X	X					X	X
12	X	X					X	X
13	X	X	X					X
14	X	X	X					X

Treatment code	Variables
A =	Control (Collect, spin, aliquot, snap-freeze at -70°C)
B =	Collect, refrigerate 4-6 hrs, spin, aliquot, freeze in -70°C freezer
C =	Collect, spin, aliquot, freeze in -20°C freezer
D =	Collect, spin, decant bulk plasma, refrigerate 48 hrs, aliquot, freeze in -70°C freezer
E =	Collect, spin, refrigerate 48 hrs (in Vacutainer), aliquot, freeze in -70°C freezer
F =	Collect, spin, decant bulk plasma, snap-freeze, thaw after 48 hrs, aliquot and refreeze
G =	Collect into Vacutainer with EDTA only, spin, aliquot, freeze in -70°C freezer
H =	Collect into Vacutainer with no PIC, spin, aliquot, freeze in -70°C freezer

Fig. 1. Plasma samples collected and treated for study. A total of 56 samples were collected, 4 from each volunteer, and samples were treated by 8 different handling variations as described *(A–H)*. All the samples collected were analyzed by EIA and 2D-PAGE (except as noted in results).

oughly evaluated for their ability to preserve proteins. We therefore designed a series of handling treatments, with conditions one might encounter in a clinical setting, to examine their effects on protein stability and to detect differences from our standard protocol (Fig. 1). We wanted to verify that our method generated stable and high quality plasma samples. The variables studied centered around the time and temperature of sample processing, the types of tubes used for sample collection, and the presence or absence of PIC.

Proteomics is generally considered to be a global analysis of all proteins in a sample. In this study, we wanted to achieve the widest view of the plasma proteins to determine if differences were present as a result of alterations in the standard protocol. We chose to use 2D-PAGE on the plasma samples in this study. In order to limit the variables to mainly the collection and storage differences, plasma samples were run with a minimum of sample preparation. Therefore, samples were not depleted of high abundance proteins (i.e., albumin and immunoglobulin) *(25–27)* , prefractionated, or run with narrow pI IPG strips *(28–30)*. The goal was to run more that 50 samples by duplicate 2D-PAGE, measure a maximum number of spots for each gel and compare the control group to each of the other groups treated by the various collection and processing parameters. For this reason we chose silver staining of the gels rather than fluorescent staining, as the latter produced fewer overall spots, thus fewer data points to evaluate. Subsequently, the gels were analyzed using classical image analysis *(31,32)* , as well as a novel approach which we term Statistical 2D-PAGE Quality Scoring (SQS). The SQS method was a semi-quantitative means of

objectively differentiating between good and bad quality gels. Different aspects and regions of the gels were scored. Finally, immunoassays for single proteins, were used as a verification method to test sample quality.

Materials and Methods

Experimental Design/Plasma Collection Protocols

Four blood samples were drawn into tubes from 14 healthy volunteers for this study yielding a total of 56 separate blood samples for proteomic analysis (Fig. 1). One sample of blood from each patient was collected in a tube that was pre-loaded with PIC and handled in accordance with our standard collection and processing protocol (control group A). For the control samples, plasma was collected in 8.0 mL lithium heparin plasma separating tubes (PST Vacutainer 367965, Becton Dickinson, Franklin Lakes, NJ) preloaded with 300 μL of PIC (stock: 2.2 mL of water added to P-2714: Sigma, St. Louis, MO). Each control tube therefore contained lithium heparin, EDTA, AEBSF, bestatin, E-64, leupeptin, and aprotinin. Following blood collection into a tube the following processing steps were conducted: (1) centrifuge at 2500*g* for 15 min at 4°C within 15 min of the draw, (2) aliquot the plasma layer within 30 min of centrifugation in 1.0 mL volumes, (3) freeze the aliquots immediately using a dry ice/alcohol bath, and (4) place frozen aliquots in –70°C freezer for storage. The goal was to have each plasma sample reach solid state within 1 h of venipuncture.

The remaining three blood samples from each volunteer were drawn and each subjected to alternate handling and storage treatments that would reasonably be encountered at research sites participating in clinical trials. Each of the groups was a variation of the control protocol and were labeled groups B through H (six samples per group). For treatment groups A through F, PIC preloaded VACUTAINERS™ were used, as described in the previous section. In treatment group B, the blood was drawn and allowed to remain at 4°C for 5 h prior to the initiation of centrifugation. The remaining steps were performed as described for the control. Treatment group C varied from the control only by changing the freezing temperature to –20°C. In treatment group D, the plasma was decanted after centrifugation, refrigerated as a bulk aliquot at 4°C for 48 h before the generation of 1.0 mL aliquots subsequent freezing at –70°C. Treatment group E was processed by storing the centrifuged VACUTAINER™ at 4°C for 48 h before aliquotting into 1.0 mL volumes and freezing. Treatment group F was the result of snap freezing the decanted bulk plasma following centrifugation, thawing after 48 h, aliquotting into 1.0 mL volumes, and re-freezing at –70°C. Treatment group G was collected into two EDTA tubes (VACUTAINER™, no. 362788, 5.0 mL, Becton Dickinson) without addition of PIC. Treatment group H excluded the PIC from the lithium heparin VACUTAINERS™ used in treatment group A, but otherwise remained unchanged.

Randomization

Samples were randomized prior to the 2D-PAGE preparation step, the IEF procedure, and the second dimension run to allow for more robust statistical analysis. Multiple operators were used for all steps of 2D-PAGE including staining and they were blinded to sample identification. Images for the SQS assessment were also randomized separately for each scorer.

Sample Analysis

Two-Dimensional Electrophoresis and Gel Imaging

Sample preparation for 2D-PAGE was conducted according to a protocol described by

the Two-Dimensional Gel Electrophoresis Laboratory of Geneva, Switzerland (http://us.expasy.org/ch2d/service/) *(33–35)* with some modification as detailed below. Each sample was aliquotted in duplicate for two 2D-PAGE gels. Briefly, a 12.5 µL aliquot of plasma was mixed with 10 µL of a solution containing 10% SDS (Fisher Scientific, Fair Lawn, NJ) and 2.3% dithioerythritol (DTE) (Sigma, St. Louis, MO). The samples were heated to 95°C for 5 min, and subsequently diluted to 500 µL with solubilization buffer containing 8*M* urea (J.T. Baker, Phillipsburg, NJ), 4% Chaps (Calbiochem, La Jolla, CA), 40 m*M* Tris (ICN Biomedicals, Aurora, OH), 65 m*M* DTE and trace Bromphenol blue (Sigma). Protein loads were based on the average plasma protein concentration of 80.0 ± 5.5 mg/mL from group A samples as measured by bicinchoninic acid (BCA) protein assay (cat. no. 23227; Pierce, Rockford, IL) (data not shown).

Isoelectric focusing was performed on 18 cm pH 3.0–10.0 NL IPG strips (Amersham, Piscataway, NJ), which were rehydrated overnight in buffer consisting of 8*M* urea, 2% Chaps 18 m*M* DTE, 2% carrier ampholytes (Amersham) and bromphenol blue at room temperature. Sixty microliters of the prepared sample was placed into a cup loader at the anodic end of the IPG strip while at both electrodes, a paper wick immersed in a solution of 3.5% DTE was placed *(26)* . Each gel consisted of 120 µg ± 7% of plasma protein. Focusing was performed, 12 strips at a time, on a Protean IEF Cell (Bio-Rad, Hercules, CA) for a total of 75kVh.

The second dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 20 × 25 cm 9–16% gradient Tris gels (Omnimatrix, Yardley, PA). The gels were run 12 at a time (Dodeca, Bio-Rad) at 100 V for 16.5 hr, followed by fixation in 40% methanol, 10% acetic acid for 1 h at 25°C. The gels were silver stained by Silver Express staining kit (cat. no. LC6100, Invitrogen, Carlsbad, CA) as described by the manufacturer.

A cooled charge coupled device (CCD) camera (Fluor-S, cat. no. 1707704; Bio-Rad) was used to digitize the gel images, using the same settings for all gels: f-stop = 5.6, exposure = 1.0 s, high resolution setting. Images were cropped to 757 by 978 pixels for uniformity and then were converted to TIFF files. These images were analyzed by Progenesis Discovery software (Version 2003.02; Nonlinear Dynamics, Durham, NC). The analysis experiment was set up for automated spot detection and matching, such that the gels from each treatment set were grouped together and an average gel image file created. The average gel from control group A was selected as the reference gel for the overall experiment with all 108 gel images. Spots were detected using the Progenesis algorithm and "Progenesis background" subtraction modes. The combined warping and matching setting was used for gel matching, as was the "cross-gel detection" which "uses matching information to make splitting more consistent within averaged gels." Unmatched spots were added to the reference gel and spot numbers synchronized. The maximum "absence of spots" allowed per treatment group was set to three. Normalized spot volumes were obtained by normalizing to the total spot volume (total density of image) after using the Intelligent Noise Correction Algorithm (INCA) for analysis. No attempt was made to manually correct mismatched spots so as to keep the objectivity of automated analysis.

Statistical 2D-PAGE Quality Scoring Analysis

The 108 randomized 2D-PAGE gel images were evaluated by five scoring analysts utilizing a questionnaire with an equal-distance scoring scale *(37)* from poor (1) to fair (3) to excellent (5). Scoring of gel images was done at random with blinded scorers (2D-PAGE

operators) to limit the subjectivity (bias). The scoring scale was used to judge nine criteria: (1) overall gel image, (2) overall focusing, (3) overall second dimension quality, (4) overall streakiness, (5) general image contrast, (6) albumin/IgG resolution, (7) cluster chain resolution, (8) low MW protein resolution, and (9) basic region resolution. The images were each scored against two reference gels, deemed to be of poor or excellent "quality." Five scorers were asked to evaluate the random-ordered gel images in sets of 20–22 per scoring session. The scorers were blinded to operator and treatment information associated with the images.

Protein Immunoassays

Enzyme immunoassay (EIA) kits were chosen based on commercial availability and applicability to plasma samples. Immunoassay kits for P-Cadherin (PCAD, cat. no. DPCD0), and human soluble Vascular Cell Adhesion Molecule-1 (sVCAM-1, cat. no. BBE3), were purchased from R & D Systems (Minneapolis, MN) and used according to the manufacturers instructions. EIAs were performed in 96-well plates for all 56 plasma samples in duplicate. Optical densities were determined by a plate reader (cat. no. 550, Bio-Rad). Concentrations of the proteins were calculated by executing a macro (XLFit, Excel, Microsoft, Redmond, WA) utilizing 4-parameter curve fits.

Statistical Analysis

Two-Dimensional Electrophoresis Scatter-Plot Analysis

The spot volume data from matched average gels were exported from Progenesis to Excel and scatter-plots were created using only spots matched by automated matching. The data was plotted on a log–log scale and linear regression analysis was done to produce a linear equation as well as the correlation coefficient (r^2).

Statistical 2D-PAGE Quality Scoring Analysis

The scores from statistical 2D-PAGE quality scoring analysis (SQS) of gel images were compiled and analyzed by the cumulative logistic regression analysis *(38)* to obtain the odds ratios for image differences by treatment effect. This gives a prediction of the probability of one treatment group scoring higher than another. All the odds ratios were compared to treatment group A. The operator and scorer effect were taken into consideration and were adjusted in the analysis.

Protein Immunoassay Analysis

The analysis of variance (ANOVA) was applied to test for differences among the treatment groups for each of the proteins measured by immunoassay. Since four blood tubes were drawn from each individual, the baseline level for proteins may be different between individuals. The baseline biological variation was adjusted for evaluating the treatment effect in the analysis to prevent confounding *(39)*.

Results

The plasma samples for this study were collected and processed according to the collection protocol shown in Fig. 1. Each sample drawn yielded at least one 1.0 mL aliquot of plasma, with most yielding three aliquots. Immunoassays were performed on all 56 samples and duplicate 2D-PAGE analysis was done on 54 samples for which there were adequate samples.

Following sample accrual and 2D-PAGE analysis, all the gel images were processed in a single analysis set by the Progenesis software. An "average gel" was created from each treatment group, which calculated average spot volumes for matched protein spots. A summary of the 2D-PAGE image spot matching analysis where spots from control group A were matched to each of the other treatment groups is shown in Table 1. For all 108 gels,

Table 1
2D-PAGE Image Analysis (Detection and Matching) for 108 Gels
Analyzed by Progenesis Discovery Software

	Treatment Groups							
	A	B	C	D	E	F	G	H
No. gels per group	26	12	12	10	12	12	12	12
No. spots per gel (range)	318–779	450–810	428–728	584–692	474–774	449–711	552–835	566–738
No. of spots matched per set (Average gels)	238	468	396	521	496	425	513	460
No. spots matched to group A spots		186	172	200	191	180	178	174

between 318 and 835 spots were found for each image. From the automated warping and matching analysis, 174 to 200 spots from the control group average gel were matched to each of the other treatment group's average gel data. Figure 2 shows the scatter-plots for each of the comparisons (control group A vs groups B through H) using only the matched spot average volumes. Variation between groups was determined by linear regression analysis and was used as a measure of sample stability. The coefficient of determination (r^2 value) from scatter-plots of raw spot volumes and INCA normalized volumes are also plotted in Fig. 2. The results suggest that treatment groups G and H, which were collected without PIC, are most different from the control group. Groups E and F also show more scatter than the other groups when correlated to the control group. As we expected, the more harshly the samples are treated, or in the absence of PIC, the more scatter is seen in the spot volume comparisons.

We followed up the previous analysis with a second analysis of the gel images in order to get another perspective on the global differences between treatment groups. A novel method was used to objectively score the quality of each gel image. Five analysts scored nine separate criteria, starting with overall gel image impression and working towards select portions of the gel (Fig. 3). The scores from the SQS process were compiled by treatment group. Each treatment group was compared to control group A. Significant odds ratios were detected from the cumulative logistic regression. Odds ratios and their 95% confidence intervals for the "Overall Gel Image" are plotted in Fig. 4. For each scoring criteria, odds ratios were compiled and those with significant differences were ranked in order (Table 2). The results indicated that treatments E, G, and H repeatedly showed significant differences from treatment A (Table 2). Even though group E samples contained PIC, the gel images from these samples ranked similarly to treatment group H samples that lacked PIC, thus leading us to conclude that it is very important to decant the plasma quickly after centrifugation. Four of the treatment groups studied (B–D, and F) showed little difference from the control group (A). These groups all contained PIC and were processed either within 6 h of the blood draw (treatments B and C) or the bulk plasma was removed from the collection tube (treatments D and F). In the

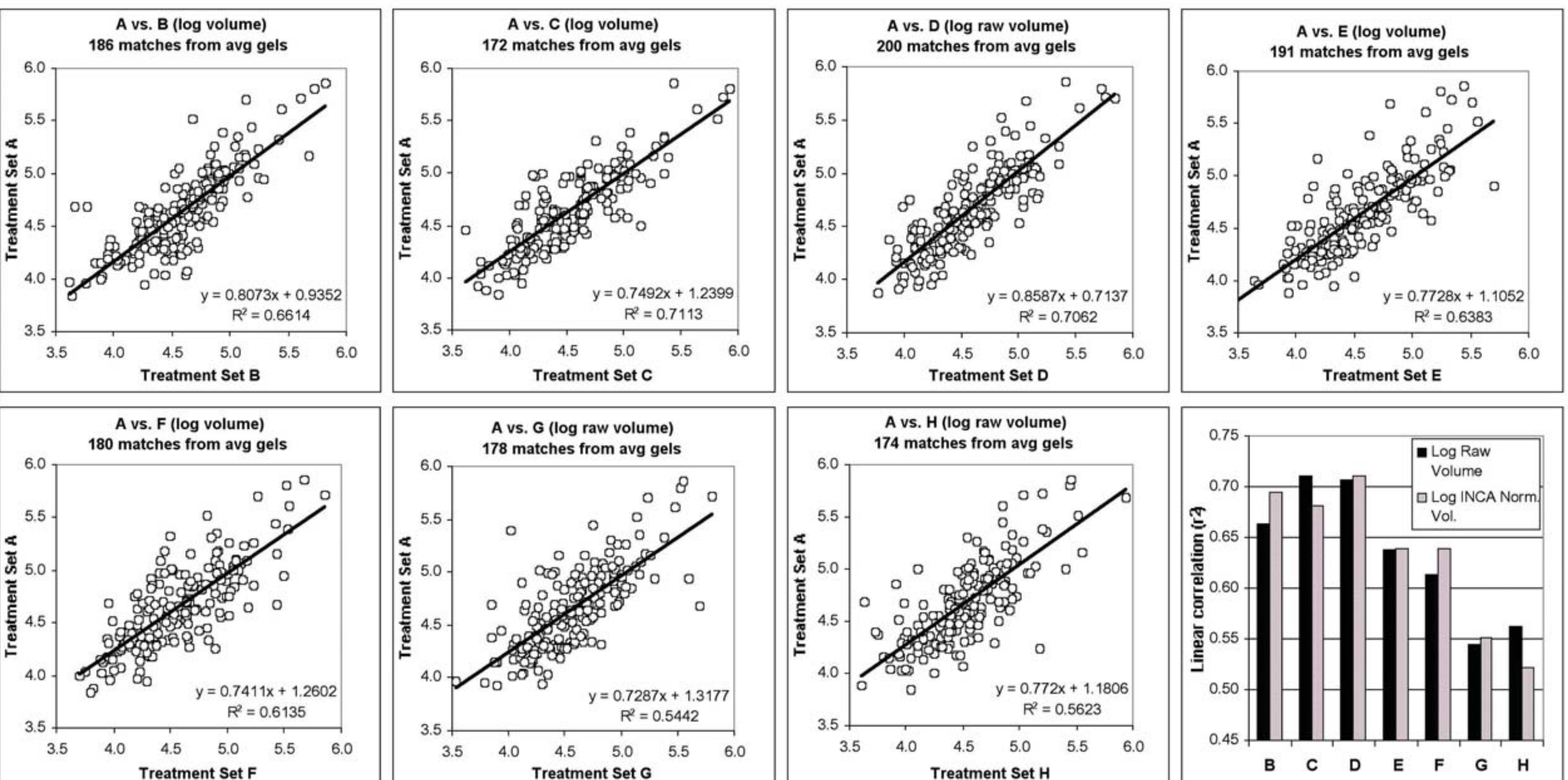

Fig. 2. Scatter-plot analysis of matched average spot volume data for each treatment group compared to control group A. Each comparison was plotted on a log–log scale to create a scatter-plot. The linear regression analysis and coefficient of determination (r^2) for each comparison is shown. Data from INCA normalized spot volume in addition to raw spot volume (shown) were plotted and the r^2 values from both sets of scatter-plots are graphed in the lower right panel.

2D Gel Scoring Checklist

General notes - Do Not compare gels to one another, Compare to the Good/Bad gel example

Please wait to score duplicate gels after the 2 individual 2D gel scoring is complete.

Utilize the 1-5 scale at right score each gel image:

Please rate using the following criteria: ***Utilizing the following scale, score each image:***

	Criteria	Description	1 Poor	2 Fair	3 Good	4 Very good	5 Excellent
1	Overall gel image	First Impression of gel, Ignore bubbles, steaks, imager artifacts	1	2	3	4	5
2	Overall focusing	Separation of spots from side to side, taking into account cup loader trail	1	2	3	4	5
3	Overall second dimension	Overall separation of spots from top to bottom	1	2	3	4	5
4	Overall streakiness	Horizontal and vertical streaking. Less streaking is better	1	2	3	4	5
5	General Contrast	Spot intensity vs background	1	2	3	4	5
6	Albumin/IgG Resolution	Look at albumin and IgG as a whole, How are the proteins resolved and How compact are the spots. Look at overall spot definition	1	2	3	4	5
7	Cluster chain Resolution	Overall all clusters surrounding albumin , Observe overall spot definition and compactness	1	2	3	4	5
8	Low MW Protein Resolution	Observe spot shape and contrast and spot count (good/bad)	1	2	3	4	5
9	Basic region Resolution	Observe overall spot definition and compactness	1	2	3	4	5

Reference Gels for Quality Scoring

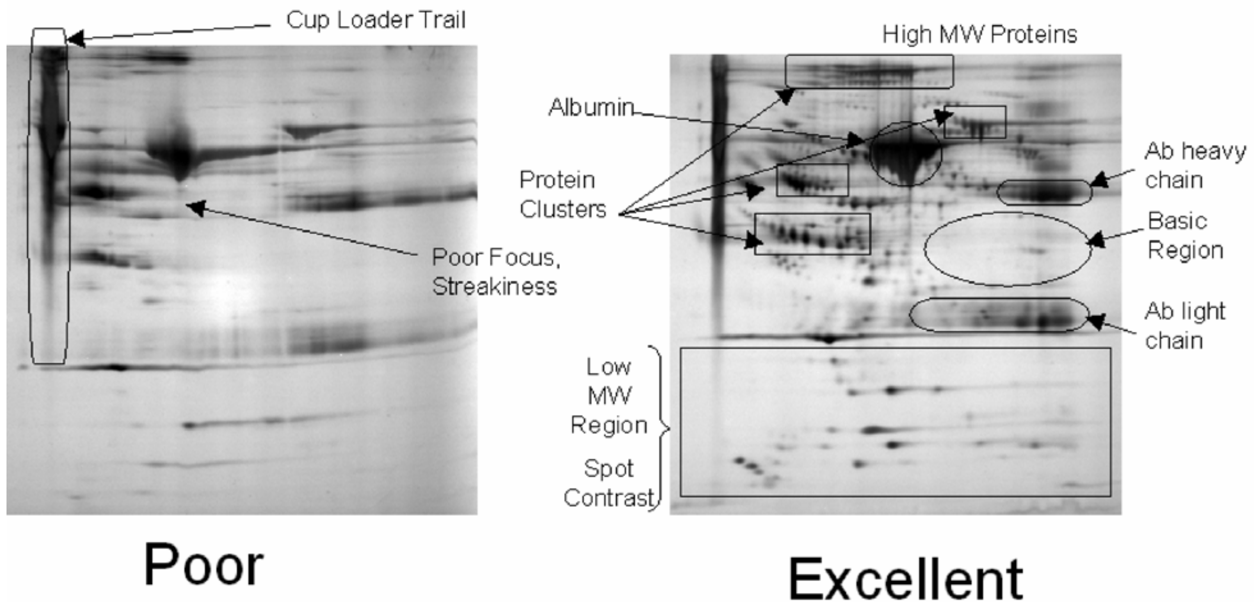

Fig. 3. 2-D Gel Quality Scoresheet used for Statistical Quality Scoring (SQS). Each of 108 gels were randomly scored by five scorers using the instructions and scoresheet shown here. Two reference gel images, one of poor and one of excellent quality, were chosen for the ends of the scoring scale by an experienced operator who did not take part in the scoring.

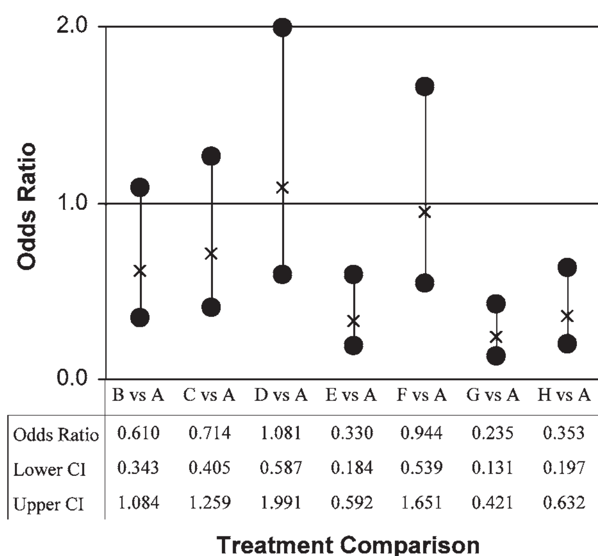

Fig. 4. The SQS scores for "Overall Gel Image" were compiled, analyzed by cumulative logistic regression and the odds ratios for each treatment group compared to control group A are plotted with a 95% confidence interval (CI). Treatment groups E, G, and H were found to be significantly different from the control group A and are ranked according to odds ratio in Table 2.

latter two conditions the bulk plasma, now containing PIC, appeared to show no differences even though samples sat at 4°C for 48 h (treatment D) or were exposed to a freeze–thaw cycle (treatment F). These data suggest that plasma collected with PIC can withstand temporary storage at 4°C, temporary storage of plasma at –20°C, or at least a single freeze–thaw cycle.

A series of immunoassays were performed on all 56 plasma samples to provide additional data to either support or refute our hypothesis. The assays were chosen because kits were commercially available and were applicable to plasma samples. Assays for Placental Cadherin (PCAD) and soluble Vascular Adhesion Molecule-1 (sVCAM-1) detected differences between treatment groups when all samples were tested. The CVs for test plasma samples collected using our standard protocol yielded CVs for intra-assay and inter-assay precision that were equivalent to those described by the manufacturer (1.4–1.9% and 4.9–5.9% respectively for PCAD and 4.3–5.9% and 8.5–10.2% respectively for sVCAM-1 [data not shown]). To limit variability, samples were thawed once, randomized and assayed in duplicate. The PCAD and sVCAM-1 immunoassay results with descriptive statistics (mean and standard error) for each treatment group are shown in Table 3. An analysis of variance (ANOVA) between control group A and each of the seven other treatment groups (B–H) were applied to the immunoassay results (Table 4). After adjusting for baseline variation, significant differences among treatments were observed. As a result, both PCAD and sVCAM-1 were shown to be present at significantly higher levels in the plasma in treatment groups G and H (no PIC) as compared with groups that were drawn with PIC present. These two proteins have been reported as potential biomarkers; PCAD has been implicated in invasive glandular lesions of the cervix and colon *(40,41)*, whereas sVCAM-1 has been linked to the development of coronary artery disease and atherosclerosis *(42,43)*.

Discussion

The overall goal of this work was to establish that our existing sample handling protocol, including the protease inhibitor cocktail, stabilized the proteins in plasma samples collected for proteomic analysis. To test this hypothesis, three different experimental analyses were used on a total of 56 human plasma samples collected from 14 separate donors. The samples were treated according to conditions specified in Fig. 1 in order to mimic conditions anticipated at clinical research sites. The analyses consisted of a classical spot analysis of large format 2D gels, a novel scoring paradigm (SQS) for the same 2D gels, and immunoassays for individual proteins.

Table 2
Ranking of Significant Differences for Each of the Nine SQS Criteria[a]

Criteria	B vs A	C vs A	D vs A	E vs A	F vs A	G vs A	H vs A
Overall Gel Image				2		1	3
Overall focusing				3		1	2
Overall Second Dimension						1	
Overall Streakiness				3		1	2
General Contrast				2		1	3
Albumin/IgG Resolution					2	1	
Cluster Chain Resolution				3		2	1
Low MW Protein Resolution				1		2	3
Basic Region resolution					2	1	

[a]Odds ratios with 95% confidence interval, like that plotted in Fig. 4, yielded significant differences which are ranked for each scoring criteria. Rank is solely based on the odds ratio estimate, not statistically tested.

Note: Groups E, G, and H consistently scored significantly different than A in most of the nine quality criteria. There were no significant differences between groups B, C, or D compared to control group A.

The challenges of 2D-PAGE and image analysis for clinical samples are many. 2D-PAGE is not a high-throughput method, and image analysis—spot detection and matching—remains less than perfect. Also, because of the wide dynamic range and presence of highly abundant proteins, plasma proteomics is a more difficult task than 2D-PAGE of bacterial extracts or even human cell extracts *(18,28)*. The classical scatter-plot analyses, that were used to compare each treatment group to the control group (Fig. 2), represent a fairly crude analysis technique. The statistical certainty was less than ideal, though there was a definite trend for samples without PIC (G and H) to be different from the control. Treatments E and F, where the samples were treated more harshly, and possibly even B showed slightly more difference than the groups C and D. While this result with image analysis supported our hypothesis, relying on the fit of a straight line to the matched spots seemed to be an inaccurate means of assessment. It was reassuring that groups G (EDTA only), and H (heparin only) showed the most differences from the control group, but it was still less than statistically rigorous.

Therefore, an alternative method for statistically assessing gel separation quality was developed, so as to find meaningful differences among treatment sets. The idea was to use the expertise of experienced 2D-PAGE operators to grade the quality of the 2D-PAGE separations, and therefore of the plasma samples themselves. The result, that of the scoresheet and randomization process described earlier (Fig. 3), were both surprisingly straightforward and reassuringly simple. Unlike the results from automated spot matching and scatter-plot analysis, the differences scored between the control group (A) and treatment groups G and H were statistically significant (Table 2). The significant difference between treatment A and E (48 h refrigeration in collection tube after centrifugation), indicates that the plasma proteome was affected through prolonged contact with the cellular material. One theory is that proteolytic enzymes excreted from leukocytes diffused through the gel plug within the blood collection tube and overwhelmed the PIC, causing proteolysis of the plasma proteins. Therefore, our collection and processing protocols now ensure that the

Table 3
Results of Immunoassays of Human Soluble Placental cCadherin (PCAD) and Human Soluble Vascular Adhesion Molecule-1 (sVCAM-1) for 56 Plasma Samples Collected for the Stability Study[a]

		Treatment Groups							
Protein	Statistics	A	B	C	D	E	F	G	H
PCAD	mean	9.203	9.451	9.022	10.088	9.645	9.030	11.094	11.494
	SD	1.691	1.615	1.742	2.217	1.875	1.582	0.908	1.367
sVCAM	mean	241.993	235.487	196.315	227.756	273.396	282.871	766.919	396.786
	SD	99.457	89.596	92.618	85.906	133.166	89.381	239.811	109.271

[a]Results for mean concentration (ng/mL) with standard deviations (SD) for each treatment group are shown.

sample tubes are centrifuged and decanted within 1 h of blood draw, and that this length of time must be closely monitored and recorded. This data implicates that biomarker "artifacts" may result from extended incubation of blood components without separation. The results of treatment groups G and H showed a benefit from the inclusion of the protease inhibitors other than just EDTA. A possible future experiment would be to test blood samples for protease activity directly (for example, using Zymogram gels). Finally, the nonsignificance among groups A–D and possibly F, indicates that the presence of PIC allowed for less expeditious processing and/or less rigorous storage conditions.

Results from gels from samples without PIC (G and H), and the samples left in a VACUTAINER™ for 48 h at 4°C (E), were clearly recognized by the SQS analysis as being distinct from the other 2D-PAGE separations. To this end, the SQS process demonstrated its usefulness. This methodology of scoring gel quality may be used for future clinical studies performed with 2D-PAGE analysis. It may also be possible to use SQS as a first pass test to determine if there are statistical differences in two sets of gels from a proteomic project prior to the use of computed image analysis. The results of SQS also proved to be readily manageable by statistical data analysis. A weighted score for each of the scoring criteria can be easily implemented to refine the SQS process to address more interesting questions.

Table 4
ANOVA Analysis (p values) of EIA Concentration Values From Each Treatment Group Compared to the Control Group

Contrast	PCAD	sVCAM-1
B vs A	0.4720	0.9812
C vs A	0.4623	0.7504
D vs A	0.2450	0.8137
E vs A	0.5575	0.6152
F vs A	0.2770	0.7109
G vs A	<0.0001	<0.0001
H vs A	<0.0001	0.0003

Recognizing that the 2D-PAGE was a global approach, we searched for individual protein differences by spot volume analysis that would match the trends seen in the global analysis, however there was too much variation in the data. By using immunoassays we did find two proteins that changed significantly by treatment. These assays proved to be a high-throughput means of testing all sam-

ples in a much shorter timeframe, and at significantly lower cost, than the 2D-PAGE method. The two proteins, PCAD and sVCAM-1, were known to be found in soluble and cell-bound forms, and we theorized that an increase in the soluble form in plasma would be because of proteolysis or cell lysis during collection and handling of the blood samples. The fact that both PCAD and sVCAM-1 were found at higher levels in treatment groups G and H vs the control group A indicates that both of these proteins are most likely cleaved by nonmetalloproteases and that the presence of the PIC substantially increases the stability of their membrane-bound forms. As surrogates for the rest of the plasma proteome, we infer that other proteins are certain to be similarly affected by the absence of protease inhibitors. The immunoassay results corroborated the various 2D-PAGE gel analysis methods, in showing that treatment groups G (EDTA only) and H (no protease inhibitors), the two groups without PIC, were again significantly different from the control group A. The increase in soluble PCAD or sVCAM-1 in these two groups suggests that they could serve as reporter proteins for proper collection or stability of plasma samples. They may be surrogate markers of plasma sample reliability.

Conclusions

As an overall assessment, each of the techniques and analysis methods showed remarkably consistent results, that our standard collection protocol, including the protease inhibitor cocktail, provides for the most stable human plasma samples for proteomic analysis. There were indications that some of the strict aliquotting and time restraints could be relaxed, given the presence of the PIC, although there was clearly a need to separate the cellular component of blood from the plasma as soon as possible, via centrifugation and decanting. Freeze–thaw cycles and temporary refrigeration both seemed to have little detrimental affect in the presence of PIC.

The goals of the experiments also drove the creation of the novel statistical scoring method (SQS). This method could be used in the future for the development of enhanced 2D-PAGE methods, both by vendors and end-users in clinical studies. It provides a robust means of differentiating subtleties in 2D-PAGE performance, and can be implemented without the need for extensive computational analysis hardware or software. We recognize that there are many new mass spectrometry technologies being used for proteomics analysis but 2D-PAGE analysis is still a common and important tool for plasma and serum profiling. Not surprisingly, immunoassays for specific proteins are a simple, fast, and inexpensive way to validate sample stability or biomarker assessment, limited only by kit availability. Finally, if one adheres to our protocol for clinical plasma collection that includes protease inhibitors, even with the wide range of collection variability in research laboratories, there appears to be little need to worry about the integrity of the samples.

Acknowledgments

The authors are grateful for the assistance of Elizabeth B. Hierl, Susan Lubin, and Lisu Wang, all of BMS-Hamilton, without whom this project would not have been possible. We are also indebted to Estelle M. Fach, Leah Ann Garulacan, Ji Gao, and Qing Xiao of BMS-Hopewell for their help running and scoring the 2D-PAGE gels. Thanks also to Thomas Colarusso and Christina Pavlick, of Nonlinear and Bio-Rad, respectively, who aided us in the area of 2D-PAGE image analysis. Dr. Kim E. Zerba helped us tremendously with the bio-

statistics. Finally, JDH thanks Patricia E. Hulmes for her valuable advice on questionnaire scoring.

References

1. Hanash S. Disease proteomics. Nature 2003; 422:226–232.
2. Hanash SM. Biomedical applications of two-dimensional electrophoresis using immobilized pH gradients: current status. Electrophoresis 2000;21:1202–1209.
3. Chambers G, Lawrie L, Cash P, Murray GI. Proteomics: a new approach to the study of disease. J Pathol 2000;192:280–288.
4. Seliger B, Kellner R. Design of proteome-based studies in combination with serology for the identification of biomarkers and novel targets. Proteomics 2002;2:1641–1651.
5. Rabilloud T. Two-dimensional gel electrophoresis in proteomics: old, old fashioned, but it still climbs up the mountains. Proteomics 2002;2:3–10.
6. Young DS, Tracy RP. Clinical applications of two-dimensional electrophoresis. J Chromatogr A 1995; 698:163–179.
7. Le Naour F, Misek DE, Krause MC, et al. Proteomics-based identification of RS/DJ-1 as a novel circulating tumor antigen in breast cancer. Clin Cancer Res 2001;7:3328–3335.
8. Rui Z, Jian-Guo J, Yuan-Peng T, Hai P, Bing-Gen R. Use of serological proteomic methods to find biomarkers associated with breast cancer. Proteomics 2003;3:433–439.
9. Zuo X, Hembach P, Echan L, Speicher DW. Enhanced analysis of human breast cancer proteomes using micro-scale solution isoelectrofocusing combined with high resolution 1-D and 2-D gels. J Chromatogr B Analyt Technol Biomed Life Sci 2002;782:253–265.
10. Charrier JP, Tournel C, Michel S, et al. Differential diagnosis of prostate cancer and benign prostate hyperplasia using two-dimensional electrophoresis. Electrophoresis 2001;22:1861–1866.
11. Charrier JP, Tournel C, Michel S, Dalbon P, Jolivet M. Two-dimensional electrophoresis of prostate-specific antigen in sera of men with prostate cancer or benign prostate hyperplasia. Electrophoresis 1999;20:1075–1081.
12. Poon TC, Johnson PJ. Proteome analysis and its impact on the discovery of serological tumor markers. Clin Chim Acta 2001;313: 231–239.
13. Steel LF, Shumpert D, Trotter M, et al. A strategy for the comparative analysis of serum proteomes for the discovery of biomarkers for hepatocellular carcinoma. Proteomics 2003;3: 601–609.
14. Kellner R, Lichtenfels R, Atkins D, et al. Targeting of tumor associated antigens in renal cell carcinoma using proteome-based analysis and their clinical significance. Proteomics 2002;2:1743–1751.
15. Sinz A, Bantscheff M, Mikkat S, et al. Mass spectrometric proteome analyses of synovial fluids and plasmas from patients suffering from rheumatoid arthritis and comparison to reactive arthritis or osteoarthritis. Electrophoresis 2002;23:3445–3456.
16. Smith MA, Bains SK, Betts JC, Choy EH, Zanders ED. Use of two-dimensional gel electrophoresis to measure changes in synovial fluid proteins from patients with rheumatoid arthritis treated with antibody to CD4. Clin Diagn Lab Immunol 2001;8:105–111.
17. He QY, Lau GK, Zhou Y, et al. Serum biomarkers of hepatitis B virus infected liver inflammation: A proteomic study. Proteomics 2003;3:666–674.
18. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics 2003; 2:50.
19. Boyanton BL, Jr., Blick KE. Stability studies of twenty-four analytes in human plasma and serum. Clin Chem 2002;48:2242–2247.
20. Flower L, Ahuja RH, Humphries SE, Mohamed-Ali V. Effects of sample handling on the stability of interleukin 6, tumour necrosis factor-alpha and leptin. Cytokine 2000;12: 1712–1716.
21. Lewis MR, Callas PW, Jenny NS, Tracy RP. Longitudinal stability of coagulation, fibrinolysis, and inflammation factors in stored plasma samples. Thromb Haemost 2001;86: 1495–1500.
22. Holodniy M, Rainen L, Herman S, Yen-Lieberman B. Stability of plasma human immunodeficiency virus load in VACUTAINER PPT plasma preparation tubes during overnight shipment. J Clin Microbiol 2000;38:323–326.
23. Marshall J, Kupchak P, Zhu W, et al. Processing of Serum Proteins Underlies the Mass

Spectral Fingerprinting of Myocardial Infarction. J Proteome Res. 2003;2:361–372.
24. Olivieri E, Herbert B, Righetti PG. The effect of protease inhibitors on the two-dimensional electrophoresis pattern of red blood cell membranes. Electrophoresis 2001;22:560–565.
25. Lollo BA, Harvey S, Liao J, et al. Improved two-dimensional gel electrophoresis representation of serum proteins by using ProtoClear. Electrophoresis 1999;20:854–859.
26. Wang YY, Cheng P, Chan DW. A simple affinity spin tube filter method for removing high-abundant common proteins or enriching low-abundant biomarkers for serum proteomic analysis. Proteomics 2003;3:243–248.
27. Rothemund DL, Locke VL, Liew A, Thomas TM, Wasinger V, Rylatt DB. Depletion of the highly abundant protein albumin from human plasma using the Gradiflow. Proteomics 2003; 3:279–287.
28. Corthals GL, Wasinger VC, Hochstrasser DF, Sanchez JC. The dynamic range of protein expression: a challenge for proteomic research. Electrophoresis 2000;21:1104–1115.
29. Herbert B, Righetti PG. A turning point in proteome analysis: sample prefractionation via multicompartment electrolyzers with isoelectric membranes. Electrophoresis 2000;21: 3639–3648.
30. Zuo X, Echan L, Hembach P, et al. Towards global analysis of mammalian proteomes using sample prefractionation prior to narrow pH range two-dimensional gels and using one-dimensional gels for insoluble and large proteins. Electrophoresis 2001;22:1603–1615.
31. Collins PJ, Juhl C, Lognonne JL. Image analysis of 2D gels: considerations and insights. Cell Mol Biol (Noisy-le-grand) 1994;40:77–83.
32. Nishihara JC, Champion KM. Quantitative evaluation of proteins in one- and two-dimensional polyacrylamide gels using a fluorescent stain. Electrophoresis 2002;23:2203–2215.
33. Hughes GJ, Frutiger S, Paquet N, et al. Plasma protein map: an update by microsequencing. Electrophoresis 1992; 13:707–714.
34. Sanchez JC, Appel RD, Golaz O, et al. Inside SWISS-2DPAGE database. Electrophoresis 1995;16:1131–1151.
35. Hoogland C, Sanchez JC, Walther D, et al. Two-dimensional electrophoresis resources available from ExPASy. Electrophoresis 1999; 20:3568–3571.
36. Hoving S, Gerrits B, Voshol H, Muller D, Roberts RC, van Oostrum J. Preparative two-dimensional gel electrophoresis at alkaline pH using narrow range immobilized pH gradients. Proteomics 2002;2:127–134.
37. Streiner DL. A checklist for evaluating the usefulness of rating scales. Can J Psychiatry 1993; 38:140–148.
38. Agresti A. Analysis of Ordinal Categorical Data. New York: John Wiley & Sons, Inc.,1984.
39. Rhodes AE, Lin E, Streiner DL. Confronting the confounders: the meaning, detection, and treatment of confounders in research. Can J Psychiatry 1999;44:175–179.
40. Han AC, Edelson MI, Peralta Soler A, et al. Cadherin expression in glandular tumors of the cervix. Cancer 2000;89:2053–2058.
41. Muller N, Reinacher-Schick A, Baldus S, et al. Smad4 induces the tumor suppressor E-cadherin and P-cadherin in colon carcinoma cells. Oncogene 2002;21:6049–6058.
42. Blankenberg S, Rupprecht HJ, Bickel C, et al. Circulating cell adhesion molecules and death in patients with coronary artery disease. Circulation 2001;104:1336–1342.
43. Cybulsky MI, Iiyama K, Li H, et al. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. J Clin Invest 2001;107:1255–1262.