Quality Control and Technical Outcome of ISO-DALT Two-Dimensional Electrophoresis in a Clinical Laboratory Setting

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In this paper, we present initial quality-control evaluations for a prototype clinical laboratory, recommendations for clinical and technical data record-keeping, and laboratory protocols designed to minimize operator-dependent analytical error in a multi-user environment. These evaluations and recommendations are based on qualitative and semiquantitative two-dimensional electrophoresis studies of the College of American Pathologists' Reference Preparation for Serum Proteins (RPSP). We include the schematic map of RPSP and manually derived estimates of analytical precision for within-run and run-to-run performance with the standard 18 × 18 cm ISO-DALT system.

Additional Keyphrases: serum protein "map" · ISO-DALT · routine use of two-dimensional electrophoresis · performance criteria · proficiency testing · reference materials · amniotic fluid · minimizing analytical variability · Clinical Evaluation Laboratory Project

High-resolution two-dimensional electrophoresis (2-D)\(^2\) is useful for the systematic characterization of complex protein mixtures, particularly those with clinical significance (1–3). This technology allows simultaneous analysis, both quantitative and qualitative, of numerous proteins of known diagnostic value in microvolume clinical specimens. In addition, 2-D enhances understanding of basic pathophysiology and allows identification of specific diagnostic "marker" proteins in diseases where a manifestation at the protein level can be inferred on the basis of inheritance patterns or on the basis of tissue pathology that reveals altered cell morphology and function. Reports of diagnosis-related protein patterns have appeared previously (4, 5).

From a medical laboratory management standpoint, adequate quality-control measures and clear quality-assurance criteria with respect to both analytical performance and experimental design are needed for clinical 2-D investigations. Currently, neither defined performance criteria nor external proficiency testing is available for 2-D laboratories. The absence of such criteria results in an overall lack of standardization that adversely affects interlaboratory data comparison, which is essential to conduct a large multicenter project such as the Human Protein Index Project (1, 2).

The Clinical Evaluations Laboratory Project is a collaborative program designed to develop and promote, through demonstration projects, quality-control procedures, experimental protocol algorithms, and standardization guidelines for medically acceptable conduct of two-dimensional electrophoresis. A prototype clinical 2-D laboratory was established in the Molecular Anatomy Program, Argonne National Laboratory, with major support from that program, Abbott Laboratories, Electro-Nucleonics, Inc., and Northwestern University Medical School. One of the purposes of this laboratory was to implement and evaluate quality-control guidelines for improvement of standardization and for objective evaluation of technical performance of 2-D with clinical specimens.

In our study we used the 18 × 18 cm ISO-DALT two-dimensional electrophoresis system developed by Anderson and Anderson (6, 7). The ISO-DALT apparatus allows 20 gels to be run simultaneously, in both the isoelectric focusing and SDS-PAGE dimensions. The data presented here summarize our first six months of operation. Our initial recommendations are based on Molecular Anatomy Program results, combined with those of others who have addressed similar questions (8, 9). Our observations are based primarily on analyses of several types of serum protein-containing specimens, including fresh serum, Reference Preparation for Serum Proteins (RPSP), and amniotic fluid.

RPSP is a certified reference material prepared from pooled human sera. We are evaluating RPSP as a potential primary standard for interlaboratory proficiency testing and quality control for 2-D. As a preliminary step we qualitatively and semiquantitatively characterized RPSP by 2-D. The schematic 2-D map of RPSP was established by analyzing 116 RPSP gels from 20 experiments. These gels were compared and contrasted with gels of fresh human sera, and differences are noted on the schematic map.

We evaluated within-run and run-to-run reproducibility by determining variation in overall spot position, linear transformation of DALT-dimension data, and local neighborhood pattern analysis. On the basis of these objective measures of gel performance, we made experimental protocol changes to help minimize operator-dependent error.

Materials and Methods

Specimens

Reference Preparation for Serum Proteins. RPSP, a certified reference material prepared and assayed under the direction of the College of American Pathologists (CAP), consists of a pool of human sera that has been processed and lyophilized. The Standards Committee of the CAP and the U.S. Centers for Disease Control cooperated in a project (10) to assign mass-concentration values for 12 clinically significant analytes contained in RPSP and in the U.S. National Reference Preparation for Human Serum (11). The 24 collaborators included experts from university clinical laboratories, commercial clinical laboratories, and commercial firms that furnish the reagents and calibrators actually

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2 Nonstandard abbreviations: 2-D, two-dimensional electrophoresis; SDS-PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; RPSP, Reference Preparation for Serum Proteins; CAP, College of American Pathologists; \(R_s\), relative electrophoretic migration; PLS (followed by number), designation used by Anderson et al. (21) for as-yet-unnamed plasma and serum proteins; XRD, x-ray-duplicating film; IgA, IgG, IgM, immunoglobulins A, G, and M, respectively; MW\(_{\text{RPSP}}\), a reference molecular mass as determined by SDS-PAGE; and MW\(_{\text{RPSP}}\), a reference molecular mass based on non-SDS-PAGE methods (e.g., ultracentrifugation, amino acid sequence, etc.).

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used in most U.S. clinical laboratories. The calibration of both RPSP and the U.S. National Reference Preparation permits conversion of mass units to international units, traceability to several other national and international reference materials, and worldwide comparability among published data. Most of the mass values are based on immunological methods, with triplicate data points for each of three dilutions. Table 1 lists the mass-concentration values for those proteins quantified in RPSP.

The vial of lyophilized RPSP was reconstituted with 1.00 mL of glass-distilled water. Aliquots of the reconstituted RPSP were stored at -70 °C.

Serum. Human serum specimens were obtained from Lauralynn Lebeck, Mount Sinai Hospital Blood Center, Chicago, IL, and from Joseph Day, Division of Support Services, Illinois Department of Law Enforcement, Maywood, IL. These specimens had had serum protein phenotypes determined. The specimens were stored at -70 °C until analysis by 2-D.

Amniotic fluid. Specimens of human amniotic fluid were kindly provided by Drs. Alice Martin and Joe Leigh Simpson, Section of Human Genetics, Northwestern University Medical School, Chicago, IL. These samples were obtained by amniocentesis during the second trimester of pregnancy. The fluids were centrifuged (5000 x g, 10 min, 4 °C) to remove cellular material, and the supernates were stored at -70 °C. Pools of five samples were prepared, and the pools, as well as the individual samples, were analyzed.

Sample Preparation

RPSP, serum, and amniotic fluid were denatured and solubilized in "SDS Mix" as described by Tollefsen et al. (12). We mixed 20 μL of RPSP or serum with 80 μL of SDS Mix, and mixed 100 μL of amniotic fluid with 100 μL of SDS Mix. All diluted samples were heated for 5 min (±15 s) at 99 °C. Eight microliters of the RPSP or serum samples, or 30 μL of the amniotic fluid samples, was applied to the isoelectric focusing (iso) gels, and the remainder of the specimens and of the denatured aliquots were stored at -70 °C.

Two Dimensional Gel Electrophoresis

The iso-DALT system (10) was used, with some minor procedural modifications designed to improve reproducibility (see Discussion). All reagents were used of "electrophoresis" grade. Iso gels contained an amphydrite mixture containing, per liter, 8 g of Servalyt pH 3–10 (lot no. 011220, Serva Feinbiochemicals), 8 g of Pharmacia's Pharmalyte pH 3–10 (lot no. 26485), and 4 g of LKB Ampholine pH 5–7 (lot no. 30). An isoelectric focusing duration of 14 000 V · h ± 1% was used throughout, with a range of 700 to 900 V. The iso gels were equilibrated (β-mercaptoethanol SDS buffer, pH 6.8) at room temperature for 10 min ± 15 s and stored at -70 °C until being loaded onto DALT gels. Iso gels were uniformly aligned to the acid end at the top of each DALT gel. Proteins were electrophoresed through the 18 x 18 cm, linear 90 g/L to 180 g/L gradient, polyacrylamide slab (DALT) gel in the usual SDS–Tris–glycine buffer (pH 8.6). The DALT gels were electrophoresed to a uniform endpoint (i.e., for an additional 30 min at 300 V after the dye front had left the bottom of the gels). Proteins in the DALT gels were stained by the ammoniacal silver-stain method of Guevara et al. (13), and XRD images were made for each silver-stained gel, as described (14).

Data Analysis

Positional scoring of the quality-control-designated protein spots in stained DALT gels was done by measuring the x and y coordinates with respect to the top and acid end of each gel. To do this, we sandwiched a transparent grid containing 2-mm divisions between each gel and a transillumination table. The x and y coordinates of the spot centers were observed and recorded, with positional assignments restricted to 0.5-mm increments, by using a 5 x lens containing a concentric spot target. The total gel lengths and widths were also recorded to the nearest 0.5 mm. The upper left-hand corner of each gel was designated as the reference origin so that positive x and y values reflected increasing pl and decreasing molecular mass.

The following procedure was used for quality-control calculations. The relative electrophoretic migration (Rm) was determined for each spot by dividing the absolute distance migrated from the top of the gel (y-coordinate) by the total length of the gel. The logit Rm (15), defined as log [Rm(1 - Rm)], was also determined for each spot. The DALT dimension data were linearized by calculating an instrument-weighted regression line (16) for each plot of log molecular mass ("MW" in the figures) vs logit Rm.

The within-run 95% confidence zones for data on the iso and DALT dimensions were plotted as spot center means ± 2 SD. Local pattern reproducibility was evaluated by comparing squares 64 x 64 mm in size (approximately 13% of the total gel area) from different gels within the same DALT run. Each square was centered about a common discrete spot (Apo Al, -1). Protein locations within the squares were analyzed by visually comparing spot patterns with respect to the center spot.

Results

Figure 1 shows a comparison of the 2-D gel patterns of fresh human serum (Frame A, pool of 11 healthy donors) and RPSP (Frame B). Figure 2 shows a schematic representation of the 2-D pattern of RPSP, compiled from examinations of many RPSP 2-D electrophoretograms. The major areas of difference between fresh serum and RPSP are highlighted by arrows in Figure 1, by symbols in Figure 2, and summarized in the text below.

Two normal serum proteins, Apo E lipoprotein and PLS:34, are diminished at least 20-fold in RPSP as compared with fresh serum, being apparent only in very heavily loaded gels. Beta-hemoglobin, which can be detected in normal serum with even trace hemolysis, is absent from RPSP. The concentrations of Apo AI lipoprotein, PLS:9, PLS:30, and PLS:32 in RPSP are also noticeably decreased as compared with fresh serum.

Two groups of protein spots are unique to RPSP. One group appears as a cluster of spots under beta-haptoglobin, and probably represents peptides produced during freezing and thawing (17). The second group is found under Apo AI

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Table 1. Concentrations of Some Constituents of the Reference Preparation for Serum Proteins (Lot No. 9628R001A)

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Conc. mg/dL</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>3597</td>
<td>7.4</td>
</tr>
<tr>
<td>α₁-Acid glycoprotein</td>
<td>57</td>
<td>20.2</td>
</tr>
<tr>
<td>α₁-Antitrypsin</td>
<td>139</td>
<td>21.9</td>
</tr>
<tr>
<td>α₂-Macroglobulin</td>
<td>187</td>
<td>10.7</td>
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<tr>
<td>Complement factor 3</td>
<td>108</td>
<td>13.3</td>
</tr>
<tr>
<td>Complement factor 4</td>
<td>22</td>
<td>19.5</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>26</td>
<td>26.2</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>105</td>
<td>34.5</td>
</tr>
<tr>
<td>IgA</td>
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</tr>
<tr>
<td>IgG</td>
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<td>9.7</td>
</tr>
<tr>
<td>IgM</td>
<td>94</td>
<td>15.5</td>
</tr>
<tr>
<td>Transferrin</td>
<td>244</td>
<td>8.1</td>
</tr>
</tbody>
</table>

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lipoprotein and retinol-binding protein, and may represent hydrolytic or proteolytic degradation products of one or both of these proteins. These data are consistent with what is expected after the multiple freeze-thaw cycles used in the manufacture of the final lyophilized material.

We evaluated 20 RPSP gels for run-to-run reproducibility in the DALT dimension by calculating the log MW-logit $R_m$ plots, as described under Methods. A representative, instrument-weighted regression line for such a log-logit plot is shown in Figure 3. Parametric statistical analyses were performed on these run-to-run regression lines ($n = 20$); the results are presented in Table 2. The coefficients of variation (CV) for the slope and intercept were 4.65% and 5.44%, respectively. The CVs for our most recent RPSP gels ($n = 10$) were 2.74% for the slope and 3.25% for the intercept. These gels were run after full implementation of the quality-assurance recommendations. Table 2 also lists the results of the statistical analysis of the within-run regression lines ($n = 19$). The CV for the log-logit regression line slope was 3.53%, and the CV for the intercept was 3.97%.

Overall variations in protein pattern were determined for two different runs, and the data were transformed into the 95% confidence neighborhoods illustrated in Figure 4. One run (Figure 4A) contained the 19 serum gels analyzed in the within-run DALT reproducibility study (Table 2). The second run (Figure 4B) contained six serum (RPSP) gels and 11 amniotic fluid gels. The spot-center mean was calculated for the ISO dimension (x-coordinate) and the DALT dimension (y-coordinate) for each spot. A 95% confidence zone was determined for each spot by plotting the within-run mean of the spot center ± 2 SD in each dimension.

Local variations in protein pattern were evaluated for the amniotic fluid gels shown in Figure 4B. Corresponding squares enclosing 13% of the gel area (see Methods) were visually analyzed for each gel. Local reproducibility was evaluated by observing the variation in the distance of a specific spot from the center spot (Apo A1, -1) in each square. These squares are displayed in Figure 5.

Discussion

The 2-D protein pattern of RPSP (lot no. 9628R001A) is very similar to that of fresh human serum. After visually analyzing 116 RPSP gels, we detected only nine substantial differences between RPSP and fresh serum (Figures 1 and 2)—differences that were consistent both from gel to gel and run to run.

We included RPSP (lot no. 9628R001A) and carbamylated creatine kinase charge standards (18, 19) in each of our 2-D experiments. The inclusion of RPSP provided a common sample for the evaluation of first- and second-dimension separations in each experiment, as well as run-to-run comparisons of identical samples. Carbamylated creatine kinase charge standards served as the primary standard for the evaluation of first-dimension separations, particularly in ampholyte optimizations.

In the DALT dimension run-to-run reproducibility studies, we calculated $R_m$ and logit $R_m$ for each quality-control designated spot. These data were linearized by calculating an instrument-weighted regression line for each plot of log MW vs logit $R_m$ (Figure 3). This sort of plot was used to achieve linearity because linear DALT gradients yield plots of MW vs mobility that are sigmoidal and thus not suitable for parametric statistical analysis.

The RPSP run-to-run CV values (4.65% for the slope and 5.44% for the intercept) indicate a level of analytical precision for the DALT dimension that is considered acceptable for clinical electrophoretic methods. Further, these results represent our earliest RPSP gels as well as our most recent. The 10 latest RPSP gels were run by several 2-D users after implementation of the quality assurance protocols. The lower CV values for these recent RPSP gels—2.74% (slope) and 3.25% (intercept) —indicate improved run-to-run precision after standardization of laboratory protocol. Visual measurements of spot-center locations on 19 serum gels from one run (Table 2) indicate good within-run precision in the DALT dimension. The CV values for the log-logit regression line were 3.53% (slope) and 3.97% (intercept). The combined data for the ISO and DALT dimensions, depicted as 95% confidence neighborhoods in Figure 4, are useful for visually assessing overall pattern reproducibility. The average protein spot size was approximately 4 mm². The 95% confidence zone was calculated as the mean of the spot center ± 2 SD for each spot, and the average ("mean") zone encompassed an area of 30.5 mm². This is a relatively
The CK charge standards (x-axis) were measured from carbamylated trains of rabbit muscle creatine kinase (18, 19) that comigrated with the RPSP. The molecular-mass scale (y-axis) was based on known serum proteins (21). Squares indicate protein spots used for quality control measurements; plus signs designate proteins found in RPSP, but not in fresh serum; diagonal lines indicate proteins found in fresh serum that are absent or substantially decreased in RPSP; dotted ellipses represent spots that are seen in very heavily loaded gels only.

small area, representing only 0.0941% of the total gel area (32 400 mm²). Simply put, this means that in 95 out of 100 runs a protein will migrate to a specific region encompassing only 0.09% of the total gel area. Therefore, these data demonstrate excellent precision in both the ISO and DALT dimensions as compared with other clinical electrophoretic methods.

The frames of Figure 4 illustrate an unexpected outcome
of the 95% confidence neighborhood plots. The data show an overall increase in DALT dimension variance for the serum protein run (Figure 4A) relative to the amniotic fluid and RPSP run (Figure 4B). The cause of the increased variance has not yet been determined, but it would have gone undetected without the neighborhood plots.

The above analyses evaluated 2-D reproducibility in an "absolute" manner via the x and y coordinate measurements. The "relative" precision for local pattern identification was evaluated in amniotic fluid gels by comparing corresponding squares enclosing 13% of the area of each gel. The squares in Figure 5 show that the local reproducibility among the 11 gels was quite good; the distance from a specific spot to the center spot varies only slightly in any square. These results, consistent with those reported by other laboratories, show that most variations in spot location are the result of a slight shift in the whole protein pattern or in a region of that pattern, rather than the isolated shifting of a single protein spot. Although the spot position may be significantly altered as determined by the "absolute" measurement (x, y coordinates), the local or "relative" protein pattern may appear unchanged.

Currently, there is no specific performance criterion for either charge or molecular-mass resolution. Based on our analyses of carbamylated CK charge standards (data not shown) and serum glycoproteins, the resolution achieved here is easily 1 electrostatic unit over a wide range of molecular weights. Charge differences of 0.5 electrostatic unit are also easily observed; however, attaining resolution of smaller charge shifts depends largely upon absolute spot size and position in the molecular-mass dimension (20). The observed resolution depends largely on the quantity of protein, the molecular-mass range of interest (because charge differences display an inverse relationship between first dimension separation and molecular mass), and experimental factors that influence separation and diffusion. Figure 1 clearly depicts the resolvability of molecules known to have microheterogeneities based on single charge shifts.
denaturation. Further, manual quality-control measurements are time-consuming and are a tedious substitute for the use of an automated gel scanner. Nevertheless, our data show that such measures are adequate for validation and objective measures of laboratory performance where cost prohibits the use of computerized image analysis or for initial analysis of performance in a new laboratory such as ours.

We believe that the good reproducibility we attained in the prototype clinical laboratory is primarily due to our close adherence to quality-assurance protocols. Specific recommendations for improved analytical performance are summarized in the following text, along with the rationale for each:

**Sample preparation.** To minimize proteolysis of the specimens, store all specimens at -70 °C, both before and after denaturation. Denature all specimens to be used in a given project with the same relative volume and the same batch of denaturant. This eliminates dilutional variability and variations arising from slight differences between lot numbers of chemicals and denaturant preparations. SDS-treated samples should be heated for 5 min ± 15 s at 95 to 99 °C. In this way heat-mediated hydrolysis and de-amidation are standardized, as are evaporation-related changes in protein concentration.

**Iso dimension.** The same optimized ampholyte mixture (including manufacturers, pH range, lot numbers, and volumes) should be used throughout an entire project. This eliminates variability resulting from differences between manufacturers and even between lot numbers of materials from the same manufacturer. Applying equivalent specimen volumes and protein content eliminates the positional shifts that are seen when these factors are varied.

We recommend using a mechanical support (sponge) at the base of the iso tubes to prevent the gels from extruding out the bottom of the tubes during isoelectric focusing. The use of a consistent focusing duration and voltage (±1%) for all experiments in a project ensures the same balance of focusing and cathodic drift in all gels.

The use of consistent equilibration conditions for all iso (10 min ± 15 s at room temperature) ensures the same degree of protein diffusion and loss. Storage-incurred diffusion and iso gel stretching may be standardized by loading all iso onto dalr gels immediately after equilibration, but this is not always convenient. Instead, we standardize these factors by storing all equilibrated iso at -70 °C before loading them onto dalr gels.

**Dalr dimension.** The same lot numbers of acrylamide and bisacrylamide should be used throughout a project, to eliminate variability arising from inter-lot differences. Positional shifts of the protein pattern due to an altered gradient density can be eliminated by using the same acrylamide gradient throughout the project. Adding the gel number labels during the gradient pouring may cause some disruption of the gradient, so we recommend installing the labels before the pour. Our gel labels are made from filter paper (Whatman no. 1) and adhere readily to the inner surface of the paraplates after being pre-moistened with glass-distilled water.

We preweigh our dry tank-buffer chemicals into packets, recalculate the total packet weights, and check the pH of the tank buffer before each electrophoresis run. This allows prompt detection of errors in the preparation of the batch-weighed buffer packets and in the preparation of the tank buffer.

Electrophoresis should be conducted at 10 °C or lower (preferably 4 to 6 °C) to minimize heat-mediated distortion of the protein patterns. We also recommend the use of a continuous temperature monitor throughout each run, to detect cooling unit and/or buffer circulating unit malfunctions. To standardize the dalr-dimension endpoint, we electrophorese for precisely 30 min at 300 V (for 20 gels) after the dye front has left the bottom of the gels. This has the added benefit of decreasing the residual ampholytes at the bottom of the gels.

**Protein detection (silver stain).** To prevent protein transfer between gels by way of surface contact, the gels must be added to the fixative individually and with agitation when stained in groups. Solution change times should be tightly monitored (±30 s) throughout the silver-stain procedure. We find that this greatly improves the reproducibility and quality of the stain.

**Data analysis and record-keeping.** We recommend that a photographic record be kept of every gel. For Coomassie-stained gels we make a 4 x 5 black-and-white negative and life-size prints. For silver-stained gels we make a positive image transparency ("XRD image") from which black-and-white negatives and prints may subsequently be prepared as necessary. We also suggest that each gel be stored in a sealed, transparent polyethylene bag containing 10 mL of dilute (5 mL/L) acetic acid. This procedure allows ease in gel handling, provides for primary data storage and minimizes microbial contamination of the gels for at least six months.

Implementation of a comprehensive technical and clinical record-keeping system is essential for compliance with the CAP's clinical laboratory accreditation guidelines. The record-keeping requirements include sections on specimen collection and handling, data management, physical facility management, a quality-control program, and a procedure manual. Examples of our data sheets for recording pertinent information about clinical specimens, sample preparation, analytical parameters for the iso and dalr dimensions, protein detection method, technical outcome, and reagent preparation and inventory are shown in Figure 6.

In conclusion, we concur with others who believe that 2-D holts great promise as a medically useful tool. Its primary advantage lies in the ability simultaneously to resolve and evaluate numerous proteins, many of them clinically significant, in small-volume samples. One genetic application where the use of 2-D may prove advantageous is the phenotyping of serum and plasma proteins. The evaluation of multiple known phenotypeable proteins currently requires many individual electrophoretic analyses, but with 2-D it is possible to determine the phenotypes of many serum or plasma proteins in a single gel, with use of only 2 mL (or less) of serum or plasma. Similarly, 2-D could be used to screen for a large number of disease-associated protein "markers" simultaneously. Finally, we believe that the level of reproducibility that we demonstrated here (Table 2, Figures 4 and 5) is acceptable for validation of the technique for use in such future routine clinical studies.

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References

3. Tracy RP, Young DS. Two-dimensional gel electrophoresis.
Fig. 6. Clinical and technical data recording sheets