

TECHNICAL IMPROVEMENTS IN 2-D GEL QUALITY AND REPRODUCIBILITY USING THE ISO-DALT® SYSTEM

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SUMMARY

The usefulness of quantitative data generated from 2-D gels depends critically on the quality of the gels. Therefore an important aspect of database development is the optimization of the 2-D analytical system itself. Here we describe a series of approaches that enhance the resolution, reproducibility and computer-analyzability of 2-D gels produced with the ISO-DALT® system. These include: 1) use of a computer-controlled gradient maker for slab gel production, 2) use of batch-standardized ampholytes selected for 2-D performance, 3) a simpler and faster method for application of first dimension gel rods onto the slab gel second dimensions, and 4) an improved Coomassie Blue staining system offering better quantitative reproducibility as determined by computer analysis. These approaches offer significant enhancements in data-generating capacity. Their evaluation through computer analysis of the resulting 2-D patterns leads us to some interesting conclusions regarding maximization of "data value", as opposed to simpler measures such as number of spots on a gel.

1 INTRODUCTION

The ISO-DALT® system for high-resolution two-dimensional electrophoresis of proteins was introduced in 1978 (1,2) and has since been widely used for systematic 2-D work. A series of evolutionary improvements has gradually increased the reliability, resolution and reproducibility of the technique (3-6) to the point of allowing long-term studies in which thousands of gels are compared.

The present paper presents a series of further improvements aimed specifically at enhancing those aspects of the gel system most critical for successful computer analysis of the 2-D data.

2 METHOD IMPROVEMENTS

2.1 A Computer-controlled Gradient Maker for Slab Gel Production

We have designed a PC-controlled peristaltic pump system ("Angelique") to allow pumping of reproducible gradients having the large volumes (1 to 3 liters) required for casting sets of 20-40 DALT slab gels. The two peristaltic pump assemblies (one for the light composition and one for the heavy) each have four channels: one each for acrylamide mix, buffer, persulfate and TEMED. Thus the pumps handle liquids which are stable, polymerization beginning only downstream at the mixing point. All four components are provided in each pump assembly so that light and heavy concentrations of each reagent (except buffer, which both pumps usually draw from one reservoir) can be independently adjusted to facilitate the required top-to-bottom polymerization regime.

Solutions delivered by the two pump assemblies are combined in a specially designed lucite block manifold and then mixed by inline "bow-tie" mixers. The output is delivered to the gel casting box by a 10 foot length of Tygon tubing. This arrangement allows the gradient maker to serve a large lab bench on which a series of casting boxes can be set up.

Each pump is driven by a stepping motor via a 5:1 reduction toothed belt and pulley system. This system, by providing for 5 motor revolutions per pump revolution, provides increased torque, increased resolution, and smoother operation. The use of toothed belts eliminates belt slippage. The two pumps are mounted on a base-plate, with the stepping motors, belts and pulleys beneath. All non-fluid-contacting mechanical components are thus protected from liquid.

The two stepping motors are driven by stepper drives which are controlled by a serial interface (RS232 serial line) from an IBM-PC-compatible computer. The software consists of a Lotus 1-2-3 spreadsheet sending control information via the Lotus Measure RS232 module. The following screens show some of the functions implemented by this software:

Figure 1. Gradients are defined by the composition of a set of linear segments. Given the concentrations of the stock solutions and the pump calibration information, the system computes pump speed ratios producing the desired %T gradient.

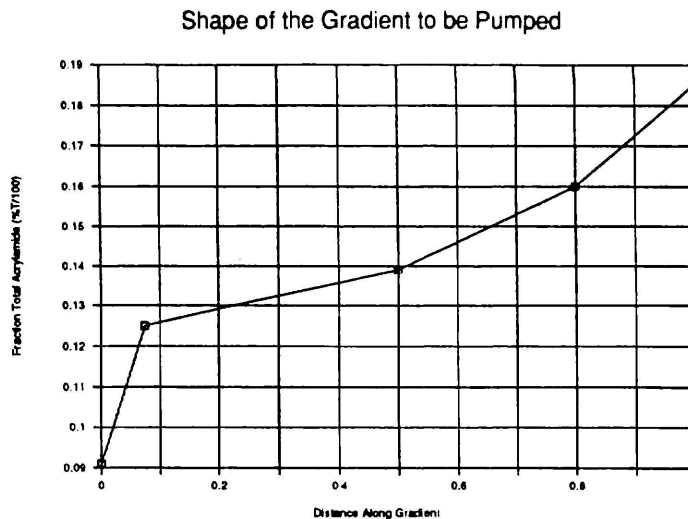


Figure 2. Screen showing gradient definition parameters:

- identifying information (name, date of entry, author and a comment describing the gradient),
- the gradient specification,
- the desired total volume,
- pump speed,
- acrylamide stock solution concentrations,
- duration of pump run,
- concentration required in the buffer stock to give the required final gel concentration, etc..

```

X1: [W15] 'R                                     READY
NEW RECALL MODIFY SAVE GRAPH PRINT QUIT
Modify the present parameters individually
      Y      Z      AA      AB      AC      AD      AE      AF
1  R
2  GRADIENT DEFINITION
3  -----
4  Gradient Name: LSB-4   Date: 21-Sep-88 Author: NLA
5  -----
6  Comment:      Linear gradient for 22 8x10 gels
7  -----
8  GRADIENT SPECIFICATION:
9  Position      AT
10 Volume: 2000 ml
11 Pump Speed: 400 ml/min      Top      0      11.0%
12                               5      11.0%
13 Light stock: 10.3%T acrylamide
14 Heavy stock: 30.8%T acrylamide      Bottom 100      18.0%
15 -----
16 Duration: 4.7 minutes      Min. AT: 6.53%
17 Buffer var: 3.5% (Top->Bottom)      Max. AT: 18.16%
18 Buffer stock: 3.3 X desired gel conc.      Span AT: 11.63%
19
20
29-Oct-88 05:53 PM                                CMD
  
```

```

X1: [W4]                                           READY
NEW SOLUTIONS LAST_USED
Enter updated identifiers for solution batches or operator
      K      L      M      N      O      P      Q      R      S
1
2  OPERATE PUMPS
3  -----
4  GRADIENT: Name: LSB-4   Date: 21-Sep-88   Author: NLA
5  -----
6  Operator: TJ           Today's Date: 29-Oct-88 17:57
7  -----
8  GRADIENT SPECIFICATION:
9  Position      AT
10 Batch # for LIGHT Acrylamide: la02-10%
11 Batch # for HEAVY Acrylamide: ha02-30.8%
12 Batch # for Buffer: bu02      Top      0      11.0%
13 L,H Para. Batches: lp01      hp01      5      11.0%
14 L,H TEMED Batches: lt01      ht01      0      0.0%
15                               0      0.0%
16 Volume: 2000 ml      Bottom 100      18.0%
17 Pump Speed: 400 ml/min
18 Light stock: 10.3%T acrylamide      Min. AT: 6.57%
19 Heavy stock: 30.8%T acrylamide      Max. AT: 18.61%
20 Duration: 4.9 minutes      Span AT: 12.03%
21
29-Oct-88 05:58 PM                                CMD          SCROLL
  
```

Figure 3. Screen showing input of solution batch specifications (kept as part of the run records).

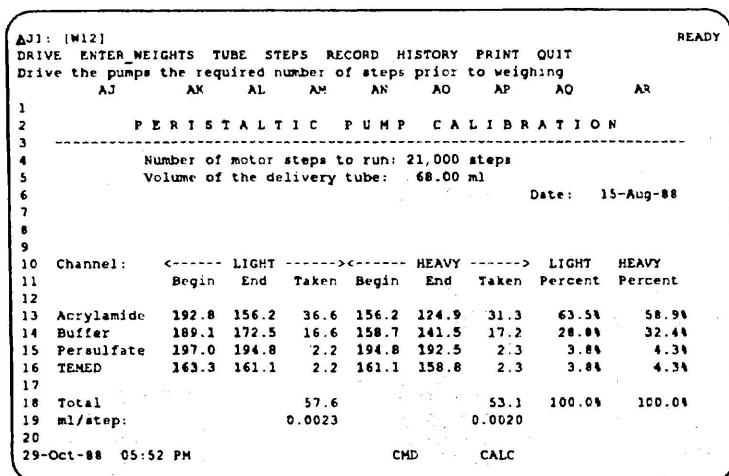


Figure 4. Screen showing the parameters input to allow calibration of the peristaltic pumps. Accurate pump calibrations are easily obtained by measuring the weight of water removed from pre-weighed vessels during a given number of pump revolutions. This information is recorded and can be plotted to reveal wear rates for the pump tubing.

Special gel casting boxes are required to allow smooth inflow of the gradient, to allow easy withdrawal of the feed tube once the gradient is in place (without liquid leaks) and to provide for the shrinkage (~2%) of the gel upon polymerization. We have developed such boxes holding 22 or 46 regular 18x18cm or 22 20x25cm DALT cassettes.

Using this system with its simplified stock solutions, it is possible to set up and cast one or more boxes of 20-40 gels in about 20 minutes (typically 4 minutes gradient pumping time). System cleanup is rapid and easy. Hence labor is reduced at the same time that reproducibility is increased. Including early prototypes, the system has been used to pour more than 20,000 gels.

2.2 Use of Batch-standardized Ampholytes Selected for 2-D Performance

We have investigated the behavior of a range of commercial ampholytes in an attempt to arrive at a logical system for routine experimental evaluation. Our objective is to identify good ampholytes for 2-D work, and to be able to detect changes in ampholyte behavior associated with lot-to-lot and longer-term variation in manufacturer's products.

In the present method we have measured the positions of CPK charge standard spots (3) at various focusing times as a means of determining smoothness and stability of the pH gradient. In principle, this method allows us to determine the positions of up to 36 protein species, more or less uniformly spaced in pI, as a function of focusing time. We have studied commercially-obtained samples of wide-range ampholytes from BioRad, Serva, LKB, Pharmacia, BDH and FMC (repackaged Serva), and of the 'semi-wide-range' BDH 4-8 product.

The following three graphs condense the information obtained for three focusing times: 9,800; 14,000; and 18,200 Volt•hours (the middle value is typi-

cally best for focusing cellular proteins). In each case the vertical axis indicates the distance from one charge standard spot (counted from the right to left starting with zero and going negative, in accordance with the charge change of the CPK variant in question) to the next in the train. The curves are shifted successively upward (by 5mm) to avoid overlap; hence only relative height within a single curve is significant. Some trains were not 'readable' to the basic end, and thus terminate before reaching the end (at -36 charges). A peak in such a curve indicates a gap in the focusing pattern. Note for instance the well-known gap in the LKB 3-10 Ampholine[®] pattern at position -17, just basic from the position of cellular actin.

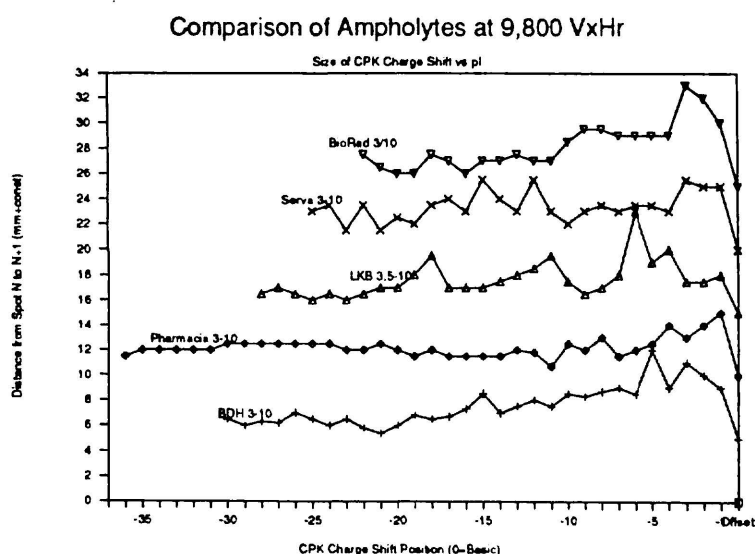


Figure 5. Patterns at 9,800 volt•hours.

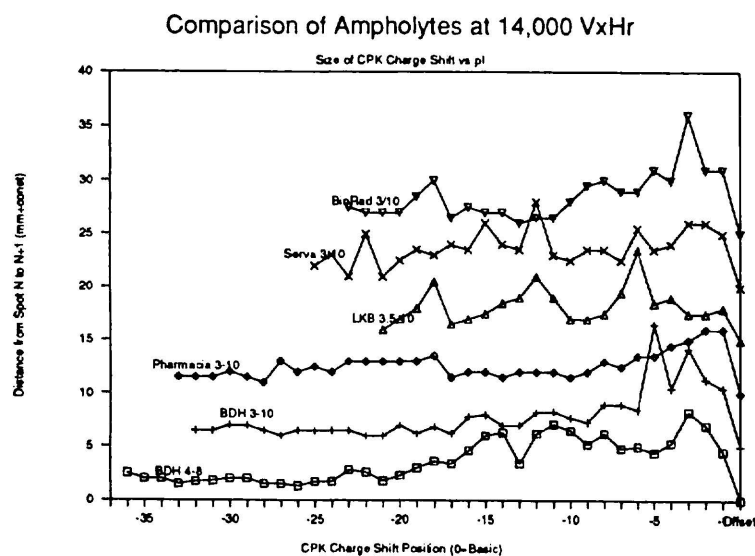


Figure 6. Patterns at 14,000 volt•hours.

Comparison of Ampholytes at 18,200 VxHr

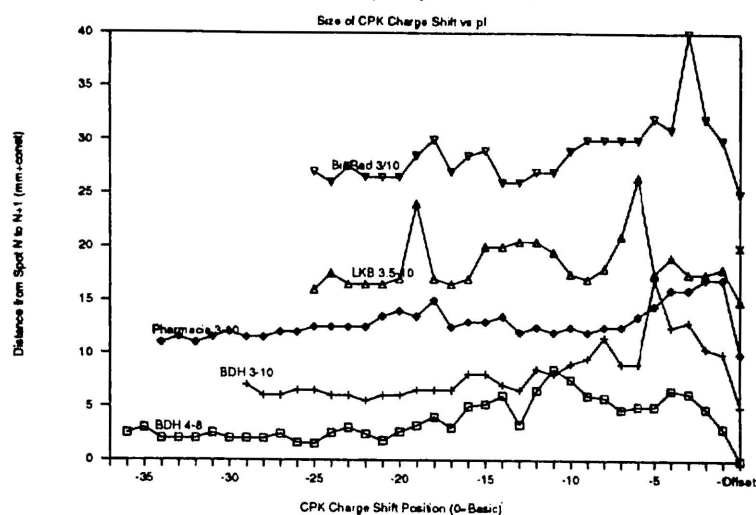


Figure 7. Patterns at 18,200 volt•hours.

Summary statistics drawn from this data are especially interesting:

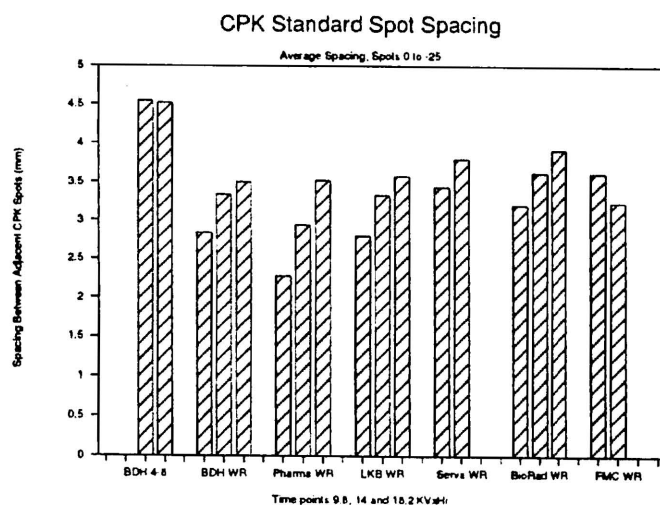


Figure 8. Average spacing of CPK charge standards as a function of focusing time.

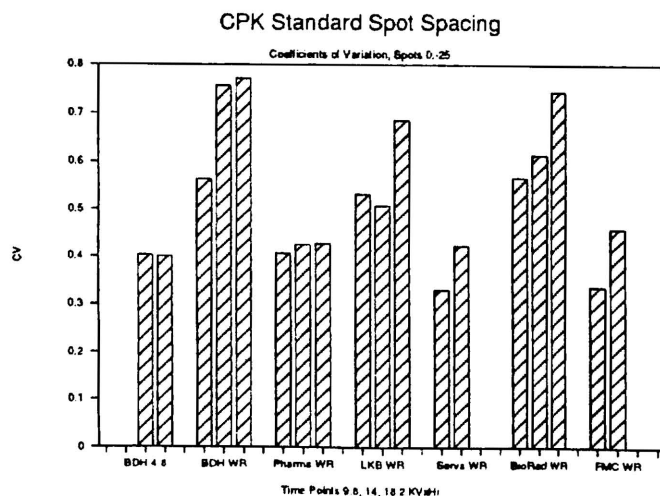


Figure 9. Standard deviations of CPK charge standard spacing as a function of time.

In the present case, we want a large average spacing with a small standard deviation (spacing as uniform as possible). In addition, we want as few major gaps as possible, since aligning spots across such gaps poses special problems in computer analysis.

At this stage in the analysis, the following general conclusions can be drawn with respect to the particular batches of material examined here: (1) the average spacing (and hence the inverse slope of the pH gradient) increases with time during the run, (2) the variation (expressed as standard deviation) among the inter-CPK-spot distances increases with focusing time substantially for some ampholytes, indicating a decrease in gradient linearity, (3) the Bio-Rad, Serva and LKB products tested exhibit some large gaps in the central part of the pattern that pose problems for computer analysis of the gels, (4) the Pharmacia wide-range product clearly generates the most linear pH gradient (if it were not for other problems associated with streaking of a class of acidic cellular proteins, Pharmalytes would appear to be best for typical 2-D work), (5) the BDH 4-8 range gives the highest average spacing with as low a CV as any product. We have concluded that the BDH 4-8 is the best for the major part of our 2-D work.

2.3 A Simpler and Faster Method for Application of First Dimension Gel Rods Onto the Slab Gel Second Dimensions

Several small 'gadgets' have been devised to facilitate DALT loading - typically the most time-consuming manual operation in the 2-D process. The first (and simplest) is the use of a special tool for manipulating the ISO gel into the DALT cassette. The tool is a piece of Teflon sheet cut in a shape rather like a French curve. Its use results in quicker loading and fewer broken gels than use of the usual thin metal spatula.

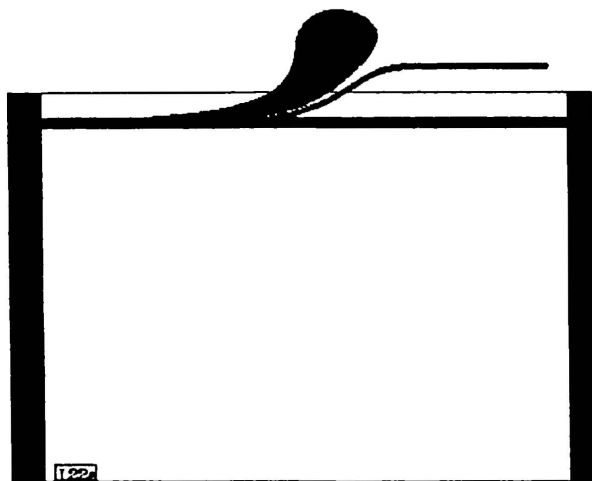


Figure 10.

The second gadget replaces agarose as a means of holding the ISO in place atop the DALT gel. Comprised of a thin stiff sheet of plastic with a pleat

of thin plastic mesh rolled over one edge, this 'wad' is slid into the cassette after ISO loading and gently pressed down, squashing the ISO against the DALT surface. Friction holds the wad in place during the run.

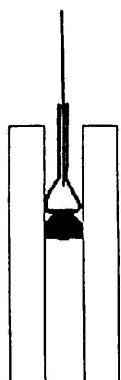


Figure 11. Side view.

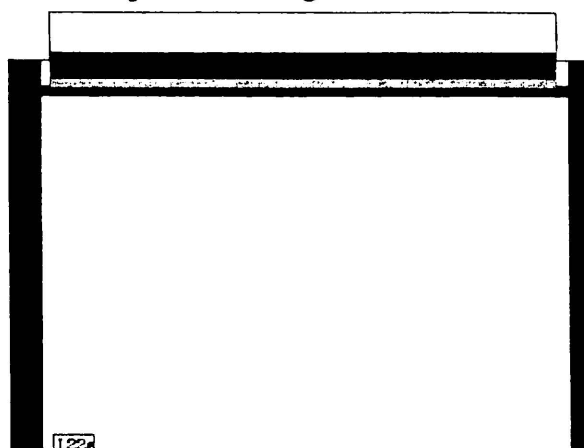


Figure 12. Front view.

Using this device, it is possible to load ISO gels directly from the tube onto the DALT (without any equilibration), seal them in place with the wad, and insert the cassette straight into the DALT tank. The entire procedure can take less than 2 minutes per gel and does not involve exposure to heat (as with an agarose overlay), allowing a substantial reduction in diffusion of low molecular mass proteins and consequent increase in overall gel resolution.

2.4 An Improved Coomassie Blue Staining System

Although the silver stain is more sensitive than Coomassie Blue, it is well known that small variations in the silver procedure can result in large changes in sensitivity and that background can be a major problem. We have therefore re-examined the potential power of Coomassie Blue-based procedures to see if detection sensitivity can be improved while retaining the superior reproducibility expected from an essentially equilibrium (as opposed to the silver's dynamic) system. We have taken as a point of departure Neuhoﬀ's work on the "colloidal" CB stain (7,8).

Potential advantages of an improved Coomassie Blue method could include: greater staining reproducibility, an almost clear background without destaining (allowing better densitometry), and greater dynamic range.

Several problems emerged in early tests of the Neuhoﬀ method: it requires large volumes of solution to equilibrate gel with methanol and ammonium sulfate in stain solution; it is difficult to apply to simple systems used for staining large sets of gels, e.g. ISO-DALT methods using 10 gels per box; it employs TCA fixation (rather than cheaper, less toxic ethanol) because of the tendency of residual ethanol to completely dissolve stain.

Our studies have led us to adopt the following system, in which the pro-

portions are adjusted to accommodate the staining of 10 gels (total gel volume about 1,000 ml) per box. Only two stock solutions are required (the fixative and the SC concentrate) neither of which contains Coomassie Blue; this is added from a salt shaker directly to the gel box.

LSB Modified Coomassie Brilliant Blue G Stain
<ul style="list-style-type: none">• Fix in 1500 ml 50% ethanol, 2% H_3PO_4 for 2 hours• Wash 3 times, each in 2000 ml water for 20 minutes• Replace water with 1500 ml of a stain solution concentrate SC:<ul style="list-style-type: none">34% methanol,17% ammonium sulfate,3% H_3PO_4• Add Coomassie Brilliant Blue powder directly to shaking box after the gels have equilibrated• Shake for 48 hours (staining essentially at equilibrium)• Transfer to cool water and shake for 30 min prior to scanning or photography.

The water washes remove ethanol that would dissolve the stain (and render the system non-colloidal, with high backgrounds). Solution SC is diluted by equilibrating with the water volume of the gels to achieve the correct final concentrations for colloidal staining automatically.

Practical advantages of this approach can be summarized as follows:

- Low, flat background makes possible computer evaluation of very small spots (max OD < 0.02).
- Up to 1500 spots can be reliably detected on many gels (e.g., mouse liver) at loadings low enough to preserve excellent resolution.
- Reproducibility appears to be very good: several hundred spots have CV < 15%. This value is at least as good as previous CB methods, and probably significantly better than many silver stain systems.
- The method is amenable to automation.

3 DISCUSSION

Taken together, the developments we report here are capable of substantially enhancing the power of 2-D gel technology in many areas of application. A bias may be noticed, however, toward improvement of those areas most critical for the generation of quantitative data through use of computer analysis systems. This approach is based on our observation, gained through the Kepler™ 2-D software

system, that once it is possible to look critically at data extracted from hundreds of gels, one finally is brought to realize how much better and more comprehensive the data from even slightly better gels would be.

4 REFERENCES

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