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INTRODUCTION

Isoelectric focusing (IEF) is a technique that separates proteins based on differences in isoelectric point (pI). In this technique, proteins migrate in a pH gradient formed by ampholytes under the influence of an electric potential until they are immobilized at a pH where their net charge is zero. Using pI standards for comparison, it is possible to accurately predict pI values of unknown protein samples. IEF can resolve peaks with differences in isoelectric point as low as 0.02 pI units, allowing for the analysis of microheterogeneity in protein samples.

Isoelectric focusing carried out in capillaries (cIEF) has attracted much interest over the last two decades. Advantages of using the capillary electrophoresis (CE) format for isoelectric focusing are: high field strengths can be applied in the capillary, due to small capillary diameters, allowing for efficient heat dissipation; on-capillary UV detection, which eliminates the need for gel staining for peak detection; and the CE platform permits automation and faster analysis times. cIEF has been established as an attractive separation technique for peptides and proteins.

The two-step process for Capillary Isoelectric Focusing involves sample focusing within the capillary followed by mobilization past a fixed detector. After introducing the sample and ampholyte mixture into a capillary, the two ends of the capillary are placed in anolyte and catholyte solutions. Generally, the anolyte and catholyte are 10-20 mM H₃PO₄ and 20-40 mM NaOH respectively. Samples are focused by applying voltage across the capillary, resulting in a pH gradient into which the proteins are resolved. Following the focusing step, sample peaks are mobilized past a detector where absorbance data is obtained. This is a critical step in the cIEF technique, since the mobilization should ideally retain the resolution achieved while focusing. Different techniques have been developed for mobilizing samples past the detector. These include chemical mobilization in which the catholyte or anolyte is replaced with a salt solution that initiates migration of the pH gradient towards the detector, hydraulic mobilization in which pressure or vacuum is applied to drive the focused zones past the detector, and a single-step process where capillary EOF is used to focus the zones and mobilize them towards the detector. The focusing and mobilization steps can be optimized to afford key improvements in resolution.

Another area where the process can be optimized is sample preparation. Key sample properties like salt content, detergents, and additives need to be carefully selected in order to maximize the potential of the technique. Choice of ampholyte is also critical since ampholytes establish the pH gradient that affects resolution of the protein in the capillary. Most cIEF separations employ a 3-10 ampholyte solution mixed with the sample to establish a pH gradient across the 3 to 10 pH range. If higher resolution is desired in a more specific pH range, “narrow-range ampholytes” can be used as
part of the mixture. For example, a 7-9 narrow-range ampholyte solution, mixed with the 3-10 ampholyte solution and sample, increases resolution in the 7-9 pH range. If heterogeneity is expected, and the protein pI falls within this range, sample peaks are more highly resolved with narrow-range ampholytes included in the mix. In these experiments, we use 8–10 or 5–7 narrow-range ampholytes in combination with 3-10 ampholytes to “zoom in” to a narrower range of the pH gradient. Using pI markers, we found the amount of ampholyte used in the mixture, in addition to focusing time and focusing voltage, greatly affect the quality of the cIEF method. Here we illustrate the importance of selecting proper conditions for an IEF experiment so that misinterpretation of results can be avoided.

**Experimental**

**Materials:** CE experiments were performed on the ProteomeLab PA 800”, and data were acquired using 32 Karat software (v. 7.0) from Beckman Coulter, Inc., Fullerton, CA. cIEF experiments were performed using the cIEF 3-10 kit (BCI part #477490, Beckman Coulter, Inc., Fullerton, CA). The cIEF kit consists of a cIEF gel, a neutral coated capillary, 3-10 ampholyte, 1 M phosphoric acid, 1 M sodium hydroxide, and four pI markers. Narrow-range ampholytes 5-7 and 8-10 were acquired to narrow the pI range (Bio-Rad; Bio-Lyte 5-7 (part #163-1152), Bio-Lyte 8-10 (part # 163-1182)). pI markers were acquired from ElphoTech, LLC (ElphoMark pI 9.6, part # P109601, ElphoMark pI 9.3, part # P109301, ElphoMark pI 8.3, part # P108301). IgG1 κ sample (purified immunoglobulin) was acquired from Sigma (Mouse Myeloma, MOPC 21, Product # M9269).

**Methods:** For use in cIEF, the narrow-range ampholyte solutions are mixed with TEMED. TEMED is used as a blocker between the most basic ampholyte and the catholyte. The narrow-range ampholyte and TEMED are mixed in a ratio of 47:3, vortexed and stored at 4°C before use. This is the narrow-range ampholyte solution (NRS).

A representative sample preparation for the cIEF run is as follows: 200 µL of cIEF gel + 5 µL 3-10 ampholyte + 15 µL 8-10 NRS solution + 0.25 µL of the 9.6 pI marker solution (20 mg/mL) + 0.25 µL of the 9.3 pI marker solution (20 mg/mL) + 1.5 µL of the 8.3 pI marker solution (15 mg/mL). During optimization, the amounts of each ampholyte along with the focusing voltage and focusing time were altered.

Mouse IgG1 κ sample is acquired as a 1.1 mg/mL solution and concentrated to 5 mg/mL using a YM-10 microcon filter. This 5 mg/mL sample is used in preparing the cIEF sample mixture as follows: 200 µL of cIEF gel + 4 µL 3-10 ampholyte + 9 µL 5-7 NRS solution + 30 µL of the 5 mg/mL IgG1 κ solution.

Prior to the cIEF run, the capillary was washed with a 10 mM phosphoric acid solution for 1 minute at 30 psi followed by a wash with deionized water for 1 minute at 30 psi. The capillary was then filled with the
sample-ampholyte-gel mixture by rinsing the capillary with the sample at 30 psi for 1.5 minutes. The two ends of the filled capillary were dipped in water in order to wash any sample adhering to their outer surface. The ends of the capillary were immersed in the anolyte and catholyte solutions and the focusing voltage was applied. The procedures for preparing anolyte and catholyte solutions are described in the Capillary Isoelectric Focusing guide included in the kit. The focusing procedure in this method calls for application of 21 kV for 10 minutes (total capillary length is 30.2 cm, field strength 700 V/cm). Focused samples are chemically mobilized by replacing the catholyte with 10 mM acetic acid.(8b) Samples are then mobilized past the detector, towards the cathode, and detected at 280 nm using a UV detector. The time program used for chemical mobilization is shown in Figure 2.

RESULTS AND DISCUSSION

We first addressed focusing time as a variable condition for being able to properly resolve a protein’s isoforms. Figure 3 illustrates a separation of a two pI mixture using cIEF under varying focusing conditions. In this experiment, we varied focusing time at a) 10 minutes b) 15 minutes and c) 20 minutes while applying a constant focusing voltage of 21 kV (field strength of 700 V/cm). The mobilization voltage (cathodic mobilization using 10 mM acetic acid as the catholyte) is also 21 kV. The 9.6 pI marker shows three peaks when mobilized for 10 minutes, suggesting it has a degree of heterogeneity. However, the marker is certified 99% pure, and with proper storage and handling should have only a limited possibility of decomposition. This made us suspect improper or inadequate focusing as the reason for the split peaks. When focusing voltage is applied for fifteen or twenty minutes, the three peaks gradually appear as a single peak (Figure 3b and 3c). This result cautions against interpretation of heterogeneity patterns in a sample peak prior to optimization of conditions for a particular sample. These data illustrate the fact that focusing can be optimized by increasing the focusing time. Twenty minutes is therefore an “optimized” focusing time for this separation, as increasing focusing time beyond 20 minutes (results not shown) provides no further change in efficiency. To further understand this, it is necessary to look at the sample composition: 200 µL cIEF gel, 5 µL 3-10 ampholyte solution, 12 µL 8-10 ampholyte solution (NRS), and a

Figure 3. Separation of pI markers 9.6 and 9.3 by cIEF (zoomed view). The focusing voltage is 21 kV (field strength 700 V/cm). Chemical mobilization is done at 21 kV with 10 mM acetic acid as the catholyte. Focusing time is different for the three overlaid electropherograms: a) 10 minute b) 15 minutes and c) 20 minutes.

Figure 4. Separation of pI markers 9.6, 9.3, and 8.3 by cIEF (zoomed view). The focusing voltage is 21 kV (field strength 700 V/cm). Samples are focused for 10 minutes. Chemical mobilization is done at 21 kV with 10 mM acetic acid as the catholyte. Difference in the two overlaid electropherograms is the amount of ampholyte added: a) Sample is a mixture of 200 µL cIEF gel + 5 µL of 3-10 cIEF ampholyte + 12 µL 8-10 NRS + 0.25 µL 9.6 and 9.3 pI marker solutions + 1.5 µL 8.3 pI marker solution. b) Identical to "a" with 3-10 ampholyte changed to 4 µL and 8-10 NRS changed to 9 µL.

Figure 5. Separation of pI markers 9.6, 9.3, and 8.3 by cIEF (zoomed view). Samples are focused for 10 minutes. Chemical mobilization is done at 21 kV with 10 mM acetic acid as the catholyte. Difference in the two overlaid electropherograms is the focusing voltage: a) focusing voltage 21 kV (700 V/cm) b) Focusing voltage 25 kV (828 V/cm).
measured amount of sample solution. In this mixture, total ampholyte amounts to ~1.6% of the total sample solution. At this concentration, longer focusing times are needed in order to focus the pH gradient and the sample peaks within the gradient. A longer focusing time therefore results in better resolved peaks. Subsequently, this implies that the peaks will focus better using shorter focusing times if less ampholyte is added to the sample mixture. We addressed this by comparing sample focusing when two different concentrations of ampholyte were used in the final sample mixture. A 5:12 ratio of 3-10:8-10 (Figure 4a) ampholyte was compared to a ratio of 4:9 (Ampholyte is ~1.2 %, Figure 4b). Both 9.6 and 9.3 pI markers focused better when the amount of ampholyte is reduced (Figure 4b). Further reduction in the amount of ampholyte did not considerably change the focusing efficiency (ratios of 4:7 and 3:6 were tried; data not shown). Reduction of the amount of ampholyte below a critical concentration will negatively affect peak resolution since there is not enough ampholyte to establish and maintain a pH gradient during focusing. With this in mind, we recommend using a ratio of 4:9 or 4:7 of 3-10 ampholyte to narrow-range ampholyte (8-10 in this case). We have observed similar results using 7-9 and 5-7 narrow-range ampholytes (data not shown).

Next, we addressed the impact of how altering focusing voltage can affect peak resolution (Figure 5). Using a 5:12 ratio of 3-10 : 8-10 ampholyte and a focusing voltage of 21 kV for 10 minutes, pI markers 9.6, 9.3, and 8.3 resolve as shown in Figure 5a. Increase in the focusing voltage to 25 kV allows for better separation (Figure 5b).

Using the results obtained in the optimization experiments for guidance, we set out to resolve charge isoforms of IgG1 κ. We decided to use a focusing voltage of 21 kV for 15 minutes and chemical mobilization at 21 kV by replacing the catholyte with 10 mM acetic acid. We used an ampholyte mixture of 3-10 and 5-7 NRS solutions mixed in a 4:9 ratio of 3-10 and 5-7 NRS solutions mixed in a 4:9 ratio. Using these conditions, we were able to separate charge isoforms with a high efficiency (Figure 6). Reproducibility of the separation pattern and mobilization time was assessed by running the same sample for three consecutive runs and showed reproducibility to be very good for this heterogeneity pattern (data not shown).

Taken together, these results suggest that optimization of focusing parameters and sample preparation is necessary while developing a method for a cIEF separation. Understanding the effects of focusing voltage, focusing time, and ampholyte concentration in the sample mixture are critical for obtaining “good” charge heterogeneity data. Selection of optimal conditions ensures reproducibility in peak patterns and improves confidence in sample heterogeneity results interpreted from the cIEF experiment.

REFERENCES:

INTRODUCTION

Capillary Isoelectric Focusing (cIEF) is a capillary electrophoresis method in which analytes are separated based on their isoelectric point (pI). Most commonly, the sample is introduced into the capillary after being mixed with ampholytes (amphoteric electrolytes), salts, and any necessary solubilizers. We refer to this as “sample mixture.” Opposite ends of the capillary and the electrodes are immersed in anolyte (a solution with acid pH) and catholyte (a solution with basic pH), respectively, and voltage is applied in order to focus ampholytes and analytes. Focusing is complete when the ampholytes establish a pH gradient in the capillary, a state associated with a minimal current in the capillary. In order to perform this CE mode properly, it is necessary to suppress or to minimize electroosmotic flow (EOF), otherwise, peaks may pass by the detector prior to focusing. This EOF suppression can be attained using static capillary coatings, like polyvinylalcohol or polyacrylamide, or by using dynamic coatings.

Two ways for performing cIEF exist: single-step cIEF or two-step cIEF. In the two-step method, there are two components of the cIEF procedure: focusing and mobilization. The final step of this mode is the mobilization of the focused analytes toward the detector. This can be performed several ways, using chemical or pressure mobilization. In chemical mobilization, a change in the composition in the anolyte or catholyte reservoir causes a shift in the pH gradient. For example, addition of a non-hydroxyl anion to the catholyte causes a reduction in hydroxyl concentration in the capillary, and thus a decrease in pH. Analytes become positively charged and migrate towards the cathode passing through the detector (see Figure 1). In pressure mobilization, the focused zones are mobilized by applying pressure from one of the ends of the capillary while maintaining voltage to compensate for band broadening caused by the parabolic profile of hydrodynamic flow.

In contrast, single-step mobilization demands a certain level of EOF in the capillary because it is this force that is used for mobilization. Thus, it can be performed in uncoated or coated capillaries in which EOF is not completely suppressed.

One of the considerations of cIEF is to avoid analytes focusing in the “blind” end of the capillary, that is, the part of the capillary past the detection point. One solution can be to block this part with a basic compound such as TEMED or another gradient extender.

One application of cIEF is to separate the different forms of proteins based on their different isoelectric points (for example, see the pioneering work done by Kilár and Hjertén,1 and in our lab2). Because of its high-resolving power, cIEF is a very powerful technique for these analyses.3 An interesting clinically significant glycoprotein is α-1-acid glycoprotein (AGP). AGP presents as different molecules depending on the amino acid sequence (it is encoded by several alleles at two loci) as well as on the glycosidic composition. The distribution of molecular AGP forms in each individual has been described to change in different types of cancer,4-7 rheumatoid arthritis,8 and other kinds of inflammation.9,10 However, few attempts to use cIEF for the separation of these forms for future studies in the biomedical field have been performed. This protein presents the added complexity of being extremely acidic having a pI of 1.8-3.811,12. This highly acidic nature complicates
analysis by cIEF. It is known that acidic proteins, which focus at the far end of a capillary, are more difficult to mobilize using chemical means than neutral and basic proteins. In addition, acidic proteins may experience anodic drift, not migrating at the same velocity as neutral and basic proteins in isoelectric focusing, giving rise to poor peak resolution or even remaining undetected. Here we investigate different approaches for the separation of AGP forms and compare them in terms of speed, resolution, and reproducibility.

**Materials and Methods**

**Chemicals and Instrumentation**

Standard human AGP was obtained from Sigma (St. Louis, MO, USA). Different combinations and percentage distribution of ampholytes of different pH ranges as well as different total percentage of ampholytes in the sample mixture were compared. The ampholyte solution of the pH range 3-5 was obtained from Bio-Rad Laboratories (Hercules, CA, USA). The solutions for pH 3-10 and 2.5-5 were obtained from Pharmacia Biotech (Uppsala, Sweden). Servalyte solution of pH range 2-4 was obtained from Serva (Heidelberg, Germany). The NaCl used in this study was from Merck (Darmstadt, Germany) and urea was acquired from Sigma. TEMED was obtained from Schwarz, Mann Biotech (Cleveland, Ohio, USA) and alanine was from Sigma. Sample mixture was prepared in cIEF gel from Beckman Coulter, Inc. (Fullerton, CA, USA). Anolyte was made up of 91 mM H₃PO₄ in cIEF gel. Catholyte was first made as 20 mM NaOH, but the method development led to the addition of different amounts of 1 M H₃PO₄ to obtain different pH values (see below). Separations were carried out in a P/ACE 5500 capillary electrophoresis system (Beckman Coulter, Inc., Fullerton, CA). Two types of capillaries were tested: eCAP Neutral capillary (polyacrylamide coated) and N-CHO Coated capillary (polyvinyl-alcohol coated), both from Beckman Coulter, Inc. Capillary length was 27 cm, i.d. 50 mm. Detection was performed at 280 nm and temperature was set at 20ºC. Polarity of the equipment will be indicated in each experiment. Separation voltage was 20 kV (both during focusing and mobilization). Conditioning of each new capillary was made by rinsing it with 10 mM H₃PO₄ (using 20 psi N₂ gas) for 2 minutes followed by a

<table>
<thead>
<tr>
<th>Method</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type of Capillary</strong></td>
<td>eCAP neutral capillary</td>
<td>Both eCAP neutral capillary</td>
<td>N-CHO coated capillary</td>
<td>N-CHO coated capillary</td>
</tr>
<tr>
<td><strong>cIEF Mode</strong></td>
<td>Single step</td>
<td>Single step</td>
<td>Single step</td>
<td>Two-step: 10 min focusing; pressure mobilization w/ voltage maintained</td>
</tr>
<tr>
<td><strong>Part of Capillary</strong></td>
<td>Short part (7 cm)</td>
<td>Short part (7 cm)</td>
<td>Long part (20 cm)</td>
<td>Long part (20 cm)</td>
</tr>
<tr>
<td><strong>Polarity Mode</strong></td>
<td>Reverse</td>
<td>Reverse</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td><strong>Gradient Extender</strong></td>
<td>TEMED</td>
<td>Alanine</td>
<td>Alanine</td>
<td>None</td>
</tr>
<tr>
<td><strong>Salts and Solubilizers</strong></td>
<td>Urea</td>
<td>Urea and NaCl</td>
<td>Urea and NaCl</td>
<td>Urea and NaCl</td>
</tr>
<tr>
<td><strong>Detailed Sample Mixture</strong></td>
<td>9.4% (v/v) ampholytes in the distribution: pH ranges 3-10, 2.5-5, 3-5, 2-4 (2.2:3:3); 5.6 M urea, 1.7% (v/v) TEMED, 1.5 mg/mL AGP</td>
<td>9.4% (v/v) ampholytes in the distribution: pH ranges 2.5-5, 3-5, 2-4 (2:4:4); 5.6 M urea, 0.21 M Alanine, 10 mM NaCl, 1.5 mg/mL AGP</td>
<td>9.4% (v/v) ampholytes in the distribution: pH ranges 2.5-5, 3-5, 2-4 (2:4:4); 5.6 M urea, 0.21 M Alanine, 10 mM NaCl, 1.5 mg/mL AGP</td>
<td>6.3% (v/v) ampholytes in the distribution: pH ranges: 2-4, 3-10, 3-5, 2.5-5 (3:1:1:1); 5.6 M urea, 20 mM NaCl, 1.5 mg/mL AGP</td>
</tr>
<tr>
<td><strong>Electropherogram in Figure:</strong></td>
<td>Fig. 3</td>
<td>Fig. 4a</td>
<td>Fig.4b</td>
<td>Fig. 6</td>
</tr>
</tbody>
</table>

Common analytical conditions: Capillary length: 27 cm; voltage applied: 20 kV; anolyte: 91 mM H₃PO₄; catholyte: 20 mM NaOH titrated to pH 11.85 with 1 M H₃PO₄; 1 M Detection: 280 nm; Temperature: 20ºC.
water rinse for 10 minutes. Capillaries were rinsed between injections (using 20 psi N₂ gas) with 10 mM H₃PO₄ for 1 minute, water for 2 minutes, and cIEF gel for 3 minutes. N-CHO capillaries were stored by rinsing 10 minutes with water and placing both ends in water at 4°C. eCAP Neutral capillaries were water rinsed for 10 minutes, rinsed with cIEF gel for 3 minutes, and stored with both ends in water at 4°C.

METHODS

Different methods were investigated to separate forms of AGP. Table 1 describes the methods tested.

RESULTS AND DISCUSSION

GENERAL REMARKS

Since AGP is a highly acidic protein leading to resolution and detection challenges described in the introduction, the reverse polarity mode, with the anolyte in the vial closer to the detection window, was the starting point for this work. This approach allows the protein forms to focus close to the point of detection, so loss of resolution inherent to mobilization over a longer distance is minimized. One type of pH instability, anodic drift, should be taken into account in cIEF of AGP. Anodic drift is a progressive loss of ampholytes into the anolyte solution. This may influence AGP separation, because being such an acidic protein, this loss of ampholytes can drag AGP sample down the reservoir. In order to avoid anodic drift in cIEF, it is recommended to use a more concentrated anolyte solution than catholyte solution. Anolyte was 91 mM H₃PO₄ and catholyte was 20 mM NaOH. However, adequate resolution of AGP bands was not obtained using these electrolytes. Based on our previous experience separating erythropoietin (EPO), we added various concentrations of phosphoric acid to the catholyte solution. With this approach, two effects were obtained: 1) “chemical mobilization” during the focusing time would counteract the opposite movement of the sample mixture towards the anode due to the anodic drift, and 2) we would avoid an independent mobilization step as it would happen from the beginning of the separation, thus performing single-step cIEF.

All the separations obtained with the methods described on Table 1 were better resolved following addition of phosphoric acid to the catholyte solution (see Figure 2 for...
an example). Results shown in Figure 2 are also important because they show how the capillary still presents EOF as mobilization occurs in the absence of chemical or pressure mobilization (Figure 2-a). We observed that higher amounts of phosphoric acid in the catholyte solution (thus, lower pH), allowed for faster AGP separation. This observation is in accordance with the proposed method of chemical mobilization towards the cathode.

Addition of the chaotropic agent urea as an AGP solubilizer was found to be necessary in all the methods tested. The use of urea in cIEF of proteins is recommended to prevent precipitation and aggregation of protein which could give rise to spikes in the electropherogram.\textsuperscript{16,17}

**CONSIDERATIONS ON THE DIFFERENT METHODS STUDIED.**

The proposed mechanism of AGP separation and mobilization in Method 1 is described graphically in Figure 1. A large number of AGP forms were separated in a short time by applying this method (Figure 3). A problem we found using this method is that TEMED degrades the capillary coating which should be stable over a pH range of 3-8, and TEMED is clearly out of this range. We noticed that when the coating was deteriorated, AGP migrated through the detector quickly with poor resolution, something we interpreted as being caused by an increase in EOF force due to degradation of the coating. To solve this problem, the gradient extender was changed to a less basic one. Amino acids, which are amphoteric molecules, were used for this. Alanine, with a pI of 6.02, was chosen since all AGP form pIs are below 6.02. Substituting alanine for TEMED, it was unnecessary to use an ampholyte range of 3-10 and only narrow-range ampholytes were used. We also found that 10 mM NaCl is necessary in the sample mixture when alanine was used as gradient extender (data not shown). These modifications to the single-step cIEF method in the reverse polarity mode are summarized in Method 2 of Table 1. The resolution of AGP obtained with this method was good considering the short analysis time (Figure 4a).

However, there was the possibility the AGP forms did not have enough distance to resolve properly, so we ran the same sample mixture in the normal polarity mode (Method 3 in Table 1) so that AGP had a longer capillary distance to resolve (Figure 4b). As a result, the resolution was markedly enhanced. In addition, in the absence of TEMED, the half life of the capillaries significantly improved. However, as shown in Figure 5, poor reproducibility was not solved. Lack of reproducibility is a known drawback to cIEF and it is possible the addition of chemical mobilization at the beginning of the separation further affected this situation. For this reason, working with alanine improved capillary half-life but had little effect on reproducibility. Results using Method 3 showed that a separation of AGP forms is possible in the long part of the capillary, in spite of the problems caused by acidity of the protein. By eliminating alanine and adding ampholytes with...
pH range 3-10 (Method 4, Table 1), a normal polarity, two-step cIEF was developed (Figure 6). Intra-day reproducibility for migration time using this two-step method (n=3) was in the range of those previously described in literature, with mean intra day RSD (%) of 0.89 (data not shown).

In summary, we applied various one and two step cIEF methods for the separation of AGP forms and, in doing so, learned advantages and drawbacks to each method. In our experience, the highest reproducibility was obtained with a normal-polarity, two-step method.

REFERENCES
1 Kilár, F., Hjertén, S. J. Chromatogr. 1989, 480, 351-357

Keeping up the P/ACE: Technical Insight Into P/ACE MDQ, ProteomeLab PA 800, and 32 Karat

RICHARD CARSON, BECKMAN COULTER, INC. FULLERTON, CALIFORNIA

RUNNING MULTIPLE MDQ INSTRUMENTS FROM A SINGLE CONTROLLER

With modular detection, the MDQ can be a powerful tool for development of new analytical methods. Once validated and transferred to quality control, sample demand on the instrument increases dramatically. Eventually, an additional instrument may be needed to keep up with throughput requirements. Addition of an instrument can involve finding laboratory space, use of additional power outlets, and a call to the IT group to hook up another controller to the network. In some cases, it may be more practical to add a second instrument to an existing controller. The purpose of this article is to describe the process of adding, configuring, and managing multiple instruments from a single controller.

PREPARING THE EXISTING CONTROLLER FOR AN ADDITIONAL INSTRUMENT

For the purpose of this article, assume an MDQ instrument is used for various analyses of small molecules. In particular, the following instrument configurations and applications are available:

- PDA detector Drug screening
- UV detector Free zone application
- UV detector Chiral analysis

For ease of use, a separate instrument configuration has been created for each application, see Figure 1.

Because we will be adding a second instrument, it is helpful to rename and group the current instrument names so they are associated with the current instruments. From the enterprise screen, change the view type to Hierarchy Pane. Right click the enterprise name in the left pane and select New Location/Group.
Instrument or system administration mode must be enabled to create groups or configure instruments. Without user login, any user has system administration privileges by default, see Figure 2.

This group will be used to identify the current instrument with the current configurations. Right click on the group name and choose a descriptive name for the current instrument (for example, Left 1 if this instrument is positioned on the left side of the bench). Highlight the enterprise in the left pane. Right click on an instrument and select cut. Right click the new group in the left pane and select paste. Repeat this for all instruments in the enterprise, see Figure 3.

As with the group name, the individual instrument names should be changed so they are associated with the current instrument. For example, Drug Screening becomes Drug Screening Left. After all instruments have been renamed, right click the group (Left 1) in the left pane and select copy. Right click the enterprise in the left pane and select paste. Provide a suitable name such as Right 2. Select the group Right 2. Rename the instruments in Right 2 as described above for the instruments in Left 1, see Figure 4.

**INSTRUMENT ID CONFIGURATION**

Do not connect the IEEE cable or turn on power to the second instrument at this time.

In the example above, the group names were chosen as Left 1 and Right 2. Each instrument can be assigned an ID from 1 to 4. All instruments ship with a default address of 1. If two instruments with the same ID are connected to the same controller many communication issues will occur. For proper operation, the ID of additional instruments must be changed to a unique ID. For this example, ID 2 will be used for the second instrument.

In group Right 2, right click on an instrument and select Configure. Click through to the detector configuration screen, see Figure 5.

DO NOT USE AUTOCONFIGURE WHEN MORE THAN ONE INSTRUMENT IS CONNECTED TO THE CONTROLLER.

Double click the detector. Observe the Device ID display at the top of the detector configuration screen. Set the device ID to 2 and click OK on all screens to accept the configuration. Repeat this process for all instruments in group Right 2.

**INSTRUMENT CONNECTION AND ID CHANGE**

IEEE communication is effective over a limited distance. To connect multiple instruments, stack the cable connections at the PC and do not chain the connections from instrument to instrument. This will limit the transmission distance to 6 feet. At this distance, although possible it is not practical to run more than two instruments on a single controller. This also reduces the risk of data overflow on the controller due to multiple instruments attempting to communicate with the controller at the same time. Disconnect the original instrument (Left 1) IEEE cable at Figure 2.

**Figure 1.**

**Figure 2.**

**Figure 3.**

**Figure 4.**
the instrument side. This will prevent configuration changes to instrument 1 while the second instrument is being configured. Prepare the second instrument with a cartridge and detector to match one of the existing instrument configurations. Connect the second instrument (Right 2) and start the instrument power. Close the cartridge and sample covers. When the instrument initialization is complete, right click the instrument icon from the Right 2 group with the same type of detector and select configure. Click through the configuration screens until the detector configuration screen is displayed.

Double click the detector icon. An hour glass is displayed while the controller looks for an instrument. When the hour glass disappears a firmware version will be displayed if a matching ID instrument is found. If NA is displayed, click Set Bus Address to change the current instrument ID. A message will be displayed to disconnect all other instruments from the system, see Figure 6.

After clicking OK, the instrument ID is set and a message is displayed to cycle the instrument power. Click OK to accept the configuration. Reconnect the first instrument IEEE cable at the first instrument to complete the setup.

**ADDITIONAL COMMENTS REGARDING MULTIPLE INSTRUMENT OPERATION**

Configuration of new instruments provides the greatest potential hazard on a multiple instrument controller. To eliminate risk during configuration changes, turn off the other instrument or disconnect the IEEE cable from the instrument.

If more than one user will be running a multiple instrument system, it is helpful to enable login and utilize projects for file management. These features enable window persistence for each user by instrument and by project.
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8th Symposium on the Practical Applications for the Analysis of Proteins, Nucleotides and Small Molecules

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