Procedures and Recommendations for Proteomics

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1. Introduction

1.1. *Overview:* The Proteomics division of the Plant-Microbe Genomics Facility utilizes the Proteome Works system from Bio-Rad. The system is based on two-dimensional separation of proteins through isoelectric focusing (IEF) for the 1st dimension (1D) via 11cm Immobilized Ph Gradient (IPG) strips followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for the 2nd dimension (2D) via precast Bio-Rad Criterion gels. Once run, the gels are visualized via two types of stains: SYPRO Ruby or BioSafe Coomassie. Alternatively, electroblotting to a variety of membranes may also be done with the 2D gels for subsequent Western blot work. The 2D protein pattern profiles for the stained gels can be analyzed by gel-to-gel comparison using PDQuest software. Finally, the facility has the capability to robotically core spots from individual gels, which can then undergo mass spectrometry analysis.

1.2. *Pre-experimental considerations*: Prior to the Facility starting any proteomic work, we require two things: (1) a free face-to-face consultation or, at the minimum, a direct phone consultation and (2) a completed proteomics order form for each new ordering of services. This initial communication is necessary to clarify the customer's goals. Furthermore, proteomics is an exacting technique in so far as it requires precision and uniformity to maximize results. In order for the Facility to best serve the customer, it is essential that the customer have a clear understanding of the proteomics service's capabilities and the input required. A critical point which must be clear is that **optimization for each sample type is almost inevitable** due to the specific characteristics of diverse samples and the requirements of individual customers. However, there are steps that can be taken during sample preparation to minimize the need for lengthy preliminary runs.

The following are guidelines and recommendations, as well as specific information, provided to maximize the value of the Facility's proteomics services. The guidelines have been provided for the benefit of the customer; they are, of course, not strict rules. The Facility is more than willing to try to accommodate variations of the following guidelines; however, in our experience these protocols work and the more one deviates from these guidelines the greater the probability of extensive optimization trials.

2. Sample Guidelines

2.1. *Sample preparation*: Due to the sensitivity of this technology, it is important to take extra measures in sample preparation. If comparative analyses are desired, it is essential that variables between samples are limited to controlled experimental modifications. For this reason, all samples that will be used for comparative analysis should be prepared in an identical manner. Many commercially available kits exist for isolation of specific fractions of protein biomass which the Facility highly recommends using if at all possible. It is also necessary, prior to sample submission, to clean up the protein samples, *i.e.* remove cell debris and other latent particulates. Ultracentrifugation (at least 100,000x g for 1hr) may be necessary unless some other method of ultrapurification is used. Regardless, this level of clean up is essential and will greatly improve proteomic results. Finally, dependent upon the extraction procedure, it may be beneficial to add nucleases to extracts to improve the 2D PAGE results.

When utilizing a custom extraction method with the initial extraction and clean up results in a whole cell extract, prefractionation of whole extracts is strongly recommended. Prefractionation aids analysis by reducing the spot pattern complexity for individual gels while **increasing the total protein detection** for the entire protein sample. There are a number of methods of prefractionation, *e.g.*, fractionation based on hydrophobicity or salt precipitation, are two examples. The type of prefractionation method used is dependent on the sample type and the goals of the individual customer.

2.2. *Sample Buffer Composition*: The IEF is performed in a loading/rehydration buffer composed of urea, CHAPS, and tributyl phosphine (TBP). Thiourea and SB3-10 may also be used depending on the nature of the protein sample. * Bio-lytes specific to the pI range of the IEF are also added to the buffer. The Facility uses two stock loading/rehydration buffers: 8M urea/ 4% (w/v) CHAPS/ 2mM TBP buffer for soluble and some insoluble (membrane associated) fractions and 7M urea/ 2M thiourea/ 2% (w/v) CHAPS/ 2% (w/v) SB3-10/ 2mM TBP for highly insoluble (membrane integral) fractions. Submitting completely solubilized samples in one of these two buffers will expedite the initial optimization and improve results. It is likely that additional sample buffer compositions will also allow efficient focusing; however, deviating from these standard buffers may result in the need for more initial optimization.

An important issue to consider for any sample buffer is the concentration of salts and other charge-carrying molecules that are present, other than the proteins. Bio-Rad specifies that the total salt concentration be below 10mM for the final loading/rehydration solution. This insures that the proteins properly respond to the current and are thus focused. During the extraction of proteins, it is inevitable that some salts are going to be extracted from the cells. It is therefore critical to try and minimize salts in the sample buffer to prevent exceeding the 10mM limit. If excessive salts are necessary during protein extraction or fractionation, additional steps, such as dialysis or gel filtration, should be taken to remove the excess salts.

* (All reagents are obtained from BioRad or Sigma.)

2.3. *Sample Concentration*: There is a standard and recommended protein load for Criterion gels when the express purpose of the gels is gel-to-gel analysis. This is due in part to the inherent

capacities of the gel system and in part to the sensitivity of the stains that are used. If an insufficient quantity of protein is loaded, the concentration of the less abundant proteins may be below the sensitivity threshold of the staining system. Therefore a relatively high protein load is necessary to observe the maximum number of protein bands. However, excessively high protein loads can result in a loss of spot resolution. Finally, for consistency between gels, accurate assay and application of equivalent protein masses is absolutely necessary.

The standard analytical protein loads for the gel systems are 50-100μg for the Criterion gels. These loads represent analytical type gels used for gel-to-gel analysis along with SYPRO Ruby staining. Protein loads for preparatory (BioSafe Coomassie staining) gels will be determined in a case by case basis due to variations between sample type and the relative concentration of individual proteins. However, the maximum protein load will likely be limited to 1-3 mg total protein load.

The 11cm IPG strip requires a final volume of 200μl for proper rehydration and loading of the strip. As mentioned above, salts can negatively impact IEF; therefore it is most convenient to prepare your sample such that the protein concentration is as high as possible. This allows the Facility to dilute the sample in loading/rehydration buffer with the goal of minimizing the ionic strength and salt concentrations. As discussed above, diluting the salt concentration in the samples will minimize the impact of latent salts and other undesired components. For this reason, protein samples should be at a minimum of 10 μg/μl total protein concentration for running analytical gels and **at least 20-25ug/μl for preparatory gel**s.

3. Choices: Flexibility of Application

3.1. *Isoelectric focusing pH range:* The IEF is performed with 11cm IPG strips from Bio-Rad which come in a number of pH ranges. Often times it is best to start out using pH 3-10, the broadest range of IPG strip. Narrower pH ranges (pH 4-7, 3-6, 5-8, and 7-10) are also available for samples containing high numbers of proteins within tight pH ranges. For extremely dense protein samples or exploration of particular protein clusters, even narrower pH ranges (pH 3.9-5.1, 4.7-5.9, 5.5-6.7, 6.3-8.3) are available. Narrowing the pH range of focusing will result in better pI resolution by further broadening the resolving distance within a pH range. It may also be possible with a narrowed pH range to load more protein on a gel, facilitating greater spot detection and improving signal levels in mass spectrometry analysis. It is important to understand that changing to different pH ranges may require some additional optimization (usually only running length variation) for each new pH range explored.

3.2. *2D gel:* As stated in section 1.1, the Facility uses Criterion Bio-rad PAGE gels. The Criterion gels are precast by Bio-Rad, and we typically start at 4-20% linear gradient gels. For narrower molecular weight ranges, gels with different percentages and gradients are available. The Criterion gels are approximately 11 cm x 8 cm in total resolving dimension and are 1 mm thick.

3.3. *Visualization of gels:* The facility can stain gels with BioSafe Coomossie stain or SYPRO Ruby protein gel stain (Bio-Rad). The Coomassie stain is a visual stain while SYPRO ruby is a fluorescent stain. The SYPRO stain, used for analytical gels, has similar levels of sensitivity with typical silver stains being slightly more sensitive. However, the SYPRO ruby stains over a greater linear range with greatly reduced background staining, lending itself to be a better

choice for in gel analysis. The Coomassie stain is not as sensitive as SYPRO thus requiring a higher protein load. For this reason, Coomassie staining is used for visualizing preparatory gels for coring protein spots for mass spectrometry analysis.

Alternatively, the Criterion gels may be used for Western blotting or other techniques that require the proteins to be on a membrane. The Facility has a Bio-Rad Criterion Blotter Cell that can transfer the proteins to a polyvinylidene difluoride (PVDF) membrane or nitrocellulose membrane. Additionally, the membranes can be stained and imaged using SYPRO Ruby membrane stain prior to hybridization procedures.

4. Gel Analysis

4.1. *pI and Molecular weight standards*: In regards to the 2nd dimension size based separation, the Criterion gels contain a molecular weight marker column. In regards to isoelectric point markers, things are more limited. It is important to stress that the PDQuest software does not provide internal pI standards for gels. However, there are methods by which standards can be introduced into the gels. One method is by doping the protein sample with known proteins and using these marker proteins to calculate molecular weight and pI for experimental proteins. This can be a very difficult method to use due to possible overlap with the experimental proteins. Another method is comparing experimental gels with separately run 2D standard gels containing pI and molecular weight standards. The major drawback for this method is the variation between gel runs, limiting this method to general information. Finally, the most reliable method is to core protein spots that appear abundant and remain constant in both the experimental and control gels. Determination of the pI and molecular weights of these proteins by N-terminal sequencing or mass spectrometry will provide internal standards for the gels, and allow for pI and molecular weight calculation of unknown spots of interest. This method may require prior knowledge of the experimental samples and will certainly require a commitment of time and resources for analysis of proteins that are common in different gels.

4.2. *Spot comparison and identification*: Upon completion of staining, the Bio-Rad PDQuest software can be used to analyze the stained gels. This analysis will determine individual protein spots on the analytical gels and compare like analytical gels to determine variances between relative protein levels under experimental conditions. This analysis can be performed by Facility staff at per hour charges, or access to a satellite license to the software can be granted by the Facility and analysis can be done by the researcher for free and at their leisure. The Facility also has the Bio-Rad Proteome Works Spot Cutter, which can selectively excise protein spots from preparative gels. The protein in the gel cores can be subsequently used for mass spectrometry analysis for specific protein identification.

It is possible to compare a single gel to a single gel, but caution should be taken. Relatively large variations between gels will often times be seen, even between duplicate gels within a single run. Therefore, it is paramount to produce a series of replicate gels to average out these variations. Otherwise, any seemly experimental differences determined through analysis could just as likely be procedural variation.