Preparation for In Gel Digestion

Preparation for *In Gel* Proteolytic Digestion and High Sensitivity Technologies

The protocol guidelines below are not a substitute for a discussion of your project. Always call to discuss the specifics of your project before submitting a sample. Successful projects stem from a thorough understanding by both the researcher and our laboratory of goals, expectations and requirements.

- **Commit as much protein as is possible**

Regardless of how sensitive our technologies are, always prep as much protein as possible. DO NOT SKIMP. If it only takes a week to generate a "one-X prep" then spend two weeks and prep 2X. All sequencing technologies, MS or Edman, are mole-based not mass based: e.g. for equal staining a 200K protein has 10X less than a 20K, of course.

- **Optimize your gel conditions so that you get the greatest amount of protein per gel volume**

Density (protein to gel volume ratio) matters. There is no restriction on the number of lanes that you send, as long as you have saturated and focused your protein in each lane. Large quantities of protein in diffuse gel volumes can fail; similarly, the way to make small amounts of protein succeed at state-of-the-art levels is to do everything you can to focus that amount in the least gel volume. (Also note this in excising your bands below.)

- **Standard SDS-PAGE or 2D gel**

Run a standard gel that optimizes for the above conditions. Always using the highest quality and fresh reagents/solvents throughout all of these procedures. Reducing, non-reducing, native or gradient conditions are OK as long as the amount of protein has been maximized and the amount of gel minimized. Please contact us if special or unusual conditions or reagents are used and note these on your form.

- **A gel thickness of 1.0 millimeter is preferred**

There is evidence that overall recovery maximizes in a gel of 1 mm thickness. Gels of 0.5mm thickness can have greater loss of protein during the stain and destain processes.

- **Stain with Coomassie Blue, standard conditions, for the minimum time to detect the protein, typically 15 - 30 min.**

Please remember that the only function of stain in this experiment is to observe your band. Therefore only stain long enough to accomplish this goal. If your protein requires five hours to visualize then so be it. However, do not stain for 5 hours if your protein is visualized in 15 min. General recipes can be used--contact us only if you need to use a very non-standard condition or reagent.

- **Destain thoroughly, standard conditions, to a clear background and nicely visualized bands**

Coomassie and other stains are interfering artifacts in our technologies. Destain long enough to clear your background and still have nicely visualized bands. All excess stain should be removed.

- **Excise bands tightly, no excess unstained or partially stained gel**

To maximize the protein to gel volume ratio, excise only the hearts of the bands. Do not include any unstained gel. For example, adding an extra 0.5mm of gel around a 1 x 10mm average band doubles its area, yet the amount of protein gained may be < 5%.

- **Pool all lanes or spots from a given protein in a single tube**

One tube per protein, even if there are multiple lanes or spots. DO NOT SPLIT protein bands into multiple tubes. Individual tubes are considered as different samples and will be processed and charged separately.

- **The tube(s) should be 1.5ml, Eppendorf-style, and in particular: plain.**

Use plain tubes. There are three items to avoid: 1) no colored tubes, 2) no O-rings and 3) no chemical treatment.

- **Also excise an *equivalent* area of the same gel that represents your background**

This should be from the same gel, processed identically to your samples. This is a blank gel to control for chemical noise and generalized, nonspecific protein background. We only need one control for each 3 to 4 proteins that are submitted.

- **Wash the gel slices in the tube 2 times with 50% acetonitrile in water.**

Each wash consists of 0.5 - 1.0 ml of 50% HPLC grade acetonitrile/water for 2 - 3 min. with gentle shaking, discarding the supernatant after each wash. This step is to normalize your gel slices for storage and shipment.

- **After discarding the supernatant from the second wash you should be left with moist gel slices or spots that are *not* submerged or swimming in any excess liquid.**

Do your best to remove all excess liquid. A drop or two is fine. The gel slices remain moist.

- **Close the cap on the tube. *Do not use parafilm.***

Parafilm can introduce chemical noise into the system. Be careful of introducing dust and surface material to the cap or lip of tube.

- **Freeze at -20 °C or below and you can store that way indefinitely until sent to us.**

Any temperature -20 °C or below is fine. Samples have been successfully processed a year later. For that reason, always identically prepare other bands from the same gel at the same time, even if you are not planning to send them. This will allow you to revisit them if they become significant at a later time.

- **Send samples to us on dry ice.**

If your shipment is international, it is your responsibility to clear all customs issues before sending. Our address is on the forms.

- **Fill out one Digestion/Separation form *for each tube* that you are sending to us, <u>including</u> the control.**

The form is your lifeline to each sample. Do not leave anything blank. We encourage you to briefly outline your goals and the biochemical significance in the instruction area. If there is information you have that is relevant to interpretation of the analysis or handling of your sample, let us know on the form! The Digestion/Separation form is the only form to fill out, but one is required <u>for each tube, including control</u>. Do NOT fill out a Protein Sequence form or Mass Spectrometry form.

- **Understand keratin contamination:**

High sensitivity analysis comes with a price: keratin contamination is always observed. Wearing gloves is not enough. In fact, the typical contamination is believed to be not your own hands but rather non-specific dust contamination being introduced in the steps after one has run the gel. Observed keratins do not necessarily have to be at the same MW as your band. Note: you cannot get rid of it but you can minimize it. Rinse all surfaces while you work, (simple rinses with HPLC grade water), that contact the gel from the point that you take the gel apart to the final storage. These surfaces include: the <u>outside</u> of your gloves, staining trays, scalpels, razor blades, tongs and the inside of the final 1.5ml tubes. Note bene: the less protein you have the more significant the non-specific keratin background will be to successful analysis of your sample. Regardless what organism you are working with, do not be surprised when keratin is observed by us.

- **Time**

***Login ("Day 0"):** Note: most delays are due to incomplete forms and billing information.*
***Week 1 (day 7-10):** In gel reduction, alkylation and proteolytic digest of 100% of the sample(s).*
***Week 2 (day 10 -18):** High sensitivity LC/MS of ~10% of the digest mixture. Purpose: Ascertain whether digest was successful. A fax will be sent that simply indicates whether we are proceeding or not. There is no detailed data analysis at this point and usually no need for a discussion.*
***Week 3 (day 15 - 25):** In depth review of acquired MS/MS spectra before consuming the remaining 90% of the digest. MS/MS sequencing by MS/MS correlation analysis and reporting.*
***Week 4 (day 20 - 30):** Discussion of project results. Further HPLC analyses and single sequence attempts, if necessary.*

- **Alternative stains.**

Colloidal Coomassie, copper, zinc and modified silver stains are compatible with the downstream technologies with significant caveats. Please discuss with us before using. Reading the literature is not a substitute for a thorough discussion with us. Remember the obvious: a more sensitive stain does not give you more protein!