NBCC Proteomics Protocol 1
Updated Version – November 28, 2018

Gel Staining by Colloidal Coomassie

1. All effort should be made to reduce keratin contamination. Make sure that you are wearing clean powder-free gloves and that any loose hair is bound. A clean lab coat may also help reduce keratin contamination.
2. Use a clean container for staining the gel. Rinse the gel container two to three times with filtered dH_20 (0.45 micron filter) to remove any dust. Add filtered dH_20 to the container.
3. Carefully place the gel into the container trying to avoid touching the gel directly where the proteins of interest are.
4. Wash the gel in filtered dH_20 for 5 to10 minutes. Wash two more times in filtered dH_20 for 5 to 10 minutes each.
5. Cover and stain the gel in Gelcode blue stain from one hour to overnight at 4^oC (do not use recycled stain solution, new solution only). Add fresh coomasie if the solution turns green after a few minutes.
6. Rinse with multiple washes of filtered dH_20 until you see decreased background. It usually takes 4x 5 minutes washes. The gel can be left covered overnight at 4^oC.
7. Take an image of the gel for your records.
8. Cover and store the gel in filtered dH_20 at 4^oC. Note that weak bands may destain over time in dH_20.

Band Cutting

9. Put on a fresh pair of powder-free gloves. Wear a lab coat (ideal is less than 2 days old) and make sure there is no gap between your coat and your gloves. Optional: put on a face mask and cap.
10. Wipe down your work area with dH_20 to eliminate dust or other potential contaminants that may be present.
11. Have the following prepared: source of very clean or disposable scalpels/blades, beaker for collecting dH_20 waste (labeled as such), plastic water bottle or beaker with fresh 1% acetic acid or 1% formic acid, microcentrifuge tubes, marker and printed image of your gel.
12. Remove most of the dH_20 from the dish containing your gel.
13. With a scalpel, cut bands out, carefully making sure that you cut as close to the band as possible to minimize the size of the gel slice. Transfer the gel slice onto a second scalpel/blade and cut the band into 3 to 4 smaller pieces.
14. Transfer the pieces to an microcentrifuge tube. Label the gel image with the same name of the tube in which you placed the band.
15. Rinse the scalpel several times with dH_20 between bands (or use a fresh scalpel). Use kimwipes to remove any visible gel material and rinse again with dH_20 if needed.
16. Add a small amount of 1% acetic acid or 1% formic acid to each tube that has gel pieces so that the pieces are covered with acid solution.
17. Clean your work area. Dispose of scalpel into sharps container, close all lids on tips, pipettes, masks etc. Wipe down any spills.

In-Gel Digest

General Tips

- Make all buffers with recently filtered (0.45-micron) dH_20.
- Minimize keratin contamination by using fresh gloves, lab coat, and opening new tip containers.
- To ensure adequate mixing and incubation, perform short vortex followed by quick centrifugation (200 rpm (30x g) for 5 sec) where appropriate.
- Watch for gel pieces that stick to pipette tips to avoid cross contamination of samples and sample losses.
- Use a CLEAN bottle of 100% ethanol to minimize risk of keratin.
- Use high-grade formic acid stock (no plastic contact!!) to make fresh 5% acid solution and replace stock solutions every month. Make sure stock solution is not expired.

Day 1

18. Prepare 100 mM Ammonium Bicarbonate (ABC) (0.08 g into 10 ml filtered dH2O, verify pH is 8.0).
19. Wash each tube 2x with 50 μl of ABC, equilibrating 10 minutes on ice between washes and aspirating to remove solutions.
20. Remove 2nd wash and add 50 μl of 95% ethanol (950 μl ethanol + 50 μl ABC, to dehydrate the gel) and incubate on ice for 20 min.
21. Remove ethanol and wash with 1x 50ul ABC. Repeat step 3.
22. Remove ethanol and add 40 μl of 5 mM DTT to reduce cysteines (1:200 dilution of 1M stock diluted in 100 mM ABC) to each sample, and incubate at 50-60°C in a water bath for 30 minutes.
23. Cool samples on ice for 5 min and remove DTT solution.
24. Dehydrate samples using 95% ethanol, and incubate on ice for 10 minutes.
25. Aspirate ethanol and add 40 μl of 50 mM iodoacetamide to each sample (1:5 dilution of 250 mM stock diluted in 100 mM ABC). Iodoacetamide is light sensitive!!
26. Place samples in a rack in a drawer for 45 minutes at room temperature.
27. Aspirate iodoacetamide, dehydrate samples using 95% ethanol, and incubate on ice for 10 minutes.
28. Aspirate ethanol and add 15 μl of trypsin (dissolve 20 μg sequence grade trypsin in 720 μl of 100 mM ABC and mix well with pipet, makes enough for 48 samples)
29. Incubate for 10 minutes at room temperature.
30. Top up reaction with 10 μl of 100 mM ABC (or enough to cover all gel pieces) and place in 37°C incubator overnight.

Day 2

31. Label fresh tubes with the corresponding names on the digestion tubes.
32. Transfer digested aqueous solution into matched labeled tubes on ice.
33. Add 30 μl of 5% formic acid to gel band digestion tube, incubate for 20 minutes on ice. This is the first extraction.
34. Vortex the gel band, quickly centrifuge (200 rpm for 5 sec) and transfer the extraction to the initial aqueous extraction on ice.
35. Add 30 μl of 5% formic acid to gel band digestion tube, incubate for 20 minutes on ice. This is the second extraction.

36. Vortex the gel band, quickly centrifuge (200 rpm for 5 sec) and transfer the second extraction to the same tube with the prior extractions.
37. Speed vac samples (no heat) to dry the combined solutions.

Reagents

It is recommended that you use these suggested reagents or reagents of equivalent grade:

Gel-Code Blue - Pierce Cat #24590 (or use homemade below)
Acetic Acid - VWR Cat #CAAX0073-59
Formic Acid - ThermoFisher Scientific Cat #14-955-319
Ammonium Bicarbonate - Bioshop Cat #AMC107
DTT - Bioshop Cat #DTT001.10 (make 1M stock in HPLC-grade H_20, store aliquots at -20^oC)
Iodoacetamide - Bioshop Cat #IOD500 (make 250 mM stock in HPLC-grade H_20, store aliquots at -20^oC (we typically make 200 μl aliquots), light sensitive)
Sequencing Grade Trypsin - Sigma Cat #T6567

Home Made Colloidal Coomassie

10% Ammonium sulfate - Bioshop Cat #05-402-7
10% Phosphoric acid - Sigma Cat #466123-25G
20% Methanol - Caledon Cat #6701-7-40
0.12% Coomassie G-250 (w/v) - Bioshop Cat #CBB555.25

1. Best if made in a dark bottle as stain is very light sensitive. The dye does not dissolve completely, so it must be mixed thoroughly before each use.

   Suggestion: keep a stir bar in the bottle and stir a few minutes before use. Solution is stable for a couple of months at 4^oC.

   The stain looks different from Pierce Gel-Code Blue when applied, but the result is equal or better in its detection limit.

2. If using silver stain instead of colloidal coomassie, it is recommended to use a kit from Pierce (https://www.thermofisher.com/order/catalog/product/24600) or the equivalent from another manufacturer.