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**Protocol status:** Working
We use this protocol and it's working

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# BAF_Protocol_001 In-gel Digestion

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## ABSTRACT

This protocol is for in-gel digestion of proteins, including large mixtures, to produce peptides for mass spectrometry analysis. The gel method can be useful for getting rid of detergents or small molecules that might interfere with minimal loss. The input is a gel band up to 1cm x 1cm. The output is a relatively clean peptide digest that is ready for quick cleanup by C18 tip.

## GUIDELINES

1. Wash hands before starting. Refrain from wearing heavy lotions or perfumes.
2. Do not wear clothing that contains wool or a lot of loose fibers.
3. Use nitrile gloves (not latex).
4. Perform all procedures under PCR clean hood cabinet.
5. All sample preparation must use polypropylene tubes cleaned by addition/removal of EtOH (pure ethanol), then $H_2O$ (water), then EtOH. Dry in clean hood for several hours upside down. This strips off polymer coating.
7. All reagents must be prepared in small glass bottles (polypropylene lined caps - no glue foil).
8. Solutions should be prepared fresh.

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**PROTOCOL integer ID:** 65051

**Keywords:** proteomics, mass spectrometry, digestion

## MATERIALS

Pre-cleaned microtubes 1.5 mL - USA scientific, SEAL-RITE® 1.5 mL MICROCENTRIFUGE TUBES color: natural.
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Screw cap microtubes 1.5 mL - Fisher Brand, Conical Screw Cap Tube, color: natural, part number: 02-681-339.
20mL liquid scintillation vials PE cone lined cap - Wheaton DWK986756
Pipette tips - Fisher Brand, color: yellow, part number: 02-681-151.
Micropipettes
ABC - Fluka analytical, Ammonium Bicarbonate, Sigma Aldrich, part number: 09830.
DTT - Fisher BioReagents, Dithiothreitol, part number: BP 172-5.
Iodoacetamide - Sigma Aldrich, Iodacetamide, part number: 1149.
Promega Trypsin: Promega, Sequencing grade modified, frozen, part number: V511C.
FA - Fisher Chemical, Formic Acid, Optima™ LC/MS Grade, part number:  A117-50.
MeOH - Fisher Chemical,  Methanol, Optima™ LC/MS Grade, part number: A456-4.
ACN - Fisher Chemical, Acetonitrile, Optima™ LC/MS Grade, part number: A955-4.
TFA - Pierce™, Trifluoroacetic Acid, part number: 28903.
Water - Fisher Chemical, Optima™ LC/MS Grade, part number: W6-4.
Syringe - Unimetrics PKS250, 250µL Peek Laboratory Syringe
2 to 20 µL Micropipette - Gilson™ F144056MT
10 to 100 C Micropipette - Gilson™ F144057MT
20 to 200 µL Micropipette - Gilson™ F144058MT
100 to 1000 µL Micropipette - Gilson™ F144059MT

BEFORE START INSTRUCTIONS

**REAGENTS**: (All reagents to be prepared fresh for each digestion)
1. Destain solution: 10 mL MeOH (methanol), 10 mL H2O (water), 500 µL Acetic Acid (glacial)
2. 100 mM ABC (ammonium bicarbonate): 0.158 g in 20 mL distilled H2O
3. 50 mM ABC: 0.079g in 20 mL distilled water
4. ACN (Acetonitrile)
5. 10mM DTT: 0.0015 g in 1 mL of 100 mM ABC (Use screw caps microtubes. DO NOT mix until directly before you are ready to use)
6. 50 mM Iodoacetamide: 0.01 g in 1 mL of 100 mM ABC (Use screw caps microtubes. DO NOT mix until directly before you are ready to use)
7. Trypsin solution: Keep on ice. Promega sequencing grade trypsin (cat. # V5113 (porcine)) dilute when ready to use in 50 mM ABC. It comes frozen in 40µL of 50 mM Acetic Acid
8. Extraction solution 1: 5% formic acid in H2O (950/50)
9. Extraction solution 2: 5% formic acid in 50% MeCN/H2O (500/450/50)

## DAY 1:

19h 30m

1 Cut gel band into small pieces (~2 mm). 10m

2 Add the pieces in a pre-cleaned 1.5 mL polypropylene tube. 5m

3 Destain the pieces in 0.5 mL destain solution for ~2hrs. 2h

4 Remove destain (using 1 gel load tip for all tubes in batch) and replace with 0.5 mL destain solution for 30min. It's okay if some stain remains. It will be removed during further wash steps. 30m

5 Remove the destain solution and dehydrate gel slices in 200 µL ACN (Acetonitrile) for 5 min. 5m

6 Remove ACN and repeat. 5m

7 Reduce the gel pieces in 30 µL of 10 mM DTT (dithiothreitol) for 0.5 h at room temperature. 30m

8 Remove the DTT solution. 1m

9 Alkylate in 30 µL 50 mM IA (iodoacetamide) at room temperature, in dark for 0.5 h. (get ice for 50 mM ABC 30m

10 Remove the IA solution. 1m

11 Wash gel pieces with 100 µL 100 mM ABC (ammonium bicarbonate) for 10 min and remove. 10m

12 Dehydrate gel pieces in 200 µL ACN for approximately 5 min. Remove ACN. 5m

13 Rehydrate gel pieces in 200 µL 100 mM ABC for 10 min. 10m

14 Remove ABC supernatant. 1m

15 Dehydrate gel slices in 200 µL ACN approximately 5 min. Remove ACN and repeat. 5m

16 Prepare trypsin. 2m
For Gel Pieces: Add 960 µL of cold 50 mM ABC to 20 ugs of Promega trypsin (Part number V5113) (it comes diluted in 40 µL of 50 mM acetic acid) on ice (20ug/mL).

17 Add 30-50 µL of the trypsin solution to cover the gel pieces and rehydrate on ice for 30 min. 30m

18 Microfuge and remove any excess trypsin solution and add 5-20 µL of 50 mM ABC. React overnight at 37°( 16h

## Day 2: 3h 40m

19 Prepare Extraction Solutions: 10m
a. Extraction Solution 1: H2O/Formic Acid (950/50)
b. Extraction Solution 2: ACN/H2O/Formic Acid (500/450/50)

20 DO NOT remove excess ABC supernatant from yesterday!!!

21 Add 10 µL Extraction Solution 1 to each tube (or more if needed to cover the gel pieces) Let rest for 10 minutes. Take off supernatant to a clean 0.5 mL tube. 10m

22 Add 10 µL Extraction Solution 2. Incubate for 10 min. Take off supernatant to the same 0.5 mL tube. 10m

23 Repeat the 10 µL Extraction Solution procedure. Take off supernatant to the same 0.5 mL tube. 10m

24 Evaporate the sample via speed vac and reconstitute to 10-100 µL total volume (depending on the amount of total protein) with 0.1% Formic acid. 2h

25 Perform desalting using C18 tips (BAF_Protocol_003): 1h
- 10 µL tips for a small amount of proteins
- 100 µL for a higher amount of proteins.