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BAF_Protocol_001a In-Gel Digestion V.2

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We use this protocol and it's working

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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 (do not use more gel as gel background rises faster than peptide signal - instead cut into sections). 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 ethyl alcohol), then H_2O, then EtOH. Dry in clean hood for several hours upside down. This strips off polymer coatings.
7. All reagents must be prepared in small glass bottles (polypropylene lined caps - no glue foil).
8. Solutions should be prepared fresh.

Materials

Pre-cleaned microtubes 1.5 mL - USA scientific, SEAL-RITE® 1.5 mL MICROCENTRIFUGE TUBES color: natural, part number:1615-5500.
Pre-cleaned microtubes 0.5 mL - USA Scientific, SEAL-RITE® 0.5 mL MICROCENTRIFUGE TUBES color: natural, part number: 1605-0020.
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
ABC - Fluka analytical, **Ammonium Bicarbonate**, Sigma Aldrich, part number: 09830.
DTT - Fisher BioReagents, **Dithiothreitol**, part number: BP 172-5.
IA - Sigma Aldrich, **Iodoacetamide**, part number: 1149.
Trypsin: Promega, Sequencing grade modified, it comes frozen (20 µg in 40µL of 50 mM Acetic Acid), 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.
EtOH - Fisher Chemical, **Ethyl Alcohol/Ethanol**, 99.5%, Millipore Sigma, part number: MEX02763.
ACN - Fisher Chemical, **Acetonitrile**, Optima™ LC/MS Grade, part number: A955-4.
Acetic Acid - Sigma-Aldrich ≥ 99.99%, part number: 338826-100 ML.
H_2O - Fisher Chemical, **Water**, Optima™ LC/MS Grade, part number: W6-4.
Syringe - Unimetrics PKS250, 250µL Peek Laboratory Syringe
Pipette tips 10 and 200 µL, CAT: 05-408-186, CAT: 02-681-151 - Fisher Brand.
Pipette tip 1000 µL, CAT: 1111-2721 - USA Scientific.
Gel-Loading tips, 1-200 µL, CAT: 02-707-81- Fisher Brand.
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
Tweezer and scalpel
White light Transilluminator, 115V, 60Hz, 1.2 Amps, part number: 95-0214-01.
Incubator chamber or Eppendorf thermomixer for temperature control.

Before start

REAGENTS: (All reagents to be prepared fresh for each digestion)

1. EtOH 75 % ethanol in distilled H_2O.
2. Destain solution: 10 mL MeOH, 10 mL H_2O, 500 µL Acetic Acid.
3. 100 mM ABC, MW: 79.08 g/M: 0.158 g in 20 mL distilled H_2O.
4. 50 mM ABC: 0.079g in 20 mL distilled H_2O.
5. 10mM DTT, MW: 154.253 g/M: 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 IA, MW: 184.962 g/M: 0.0092 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: Take out the enzyme from -20C and keep it on ice just before two steps to be use.
8. Extraction solution 1: 5% FA in H_2O. 10 mL volume (Solution can be use for a couple weeks)
9. Extraction solution 2: 5% FA in 50% ACN/H_2O. 10 mL volume

*Buffers may keep at RT for up to two weeks but reagents such as DTT, IA and trypsin must be fresh right before use.

DAY 1:

19h 30m

1	Clean tweezer and scalpel with EtOH 75%. Spread EtOH on the transilluminator area you will use to cut the bands and clean it up by sliding the kimwipes in only one direction.	10m
2	Cut gel band with the scalpel into small pieces (~2 mm) on the transilluminator.	
3	Transfer the pieces with the scalpel/tweezer in a pre-cleaned 1.5 mL polypropylene tube.	5m
4	Add 500 µL of destain solution to the pieces. Let rest for ~2hrs at RT (Room Temperature).	2h
5	Discard destain (using 1 gel load tip for all tubes in batch) and replace with another 500 µL of destain solution. Let rest for 30min. It's okay if some stain remains. It will be removed during further wash steps.	30m
6	Discard the destain solution and dehydrate gel slices by adding 200 µL ACN. Let rest for 5 min.	5m
7	Discard ACN and repeat step 6 dehydration.	5m
8	Reduce the proteins by adding 30 µL of 10 mM DTT. Incubate for 30 min at RT.	30m
9	Discard the DTT solution.	1m
10	Alkylate the proteins by adding 30 µL of 50 mM IA. Incubate it for 30 min at RT, in dark. (get ice and keep the 50 mM ABC solution on it).	30m
11	Discard the IA solution.	1m
12	Wash gel pieces with 100 µL of 100 mM ABC for 10 min and discard the solution.	10m
13	Dehydrate gel pieces in 200 µL ACN for approximately 5 min and discard the ACN.	

5m

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

15 Discard ABC solution. 1m

16 Dehydrate gel slices in 200 µL ACN approximately 5 min. Discard ACN and repeat this step. (Take out the Trypsin from -20C and keep it on ice). 5m

17 Prepare trypsin at (20µg/mL) by adding 960 µL cold 50 mM ABC. 2m

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

19 Microfuge and remove any excess trypsin solution and add 5-20 µL of 50 mM ABC. Incubate the reaction overnight at 37°C. 16h

Day 2: 3h 40m

20 Prepare Extraction Solutions: 10m
a. Extraction Solution 1: H2O/Formic Acid: 9.5 mL H_2O/0.5 mL FA
b. Extraction Solution 2: ACN/H2O/Formic Acid: 5 mL ACN/4.5 mL H_2O/0.5 mL FA

21 **DO NOT remove excess ABC supernatant from yesterday!!!**

22 Add 10 µL Extraction Solution 1 to each tube (or more if needed to cover the gel pieces). Let rest for 10 minutes. Transfer the supernatant to a pre-cleaned 0.5 mL tube. 10m

23 Add 10 µL Extraction Solution 2. Let rest for 10 min. Transfer the supernatant to the same 0.5 mL tube. 10m

24 Repeat the 10 µL Extraction Solution procedure (steps 22-23). Transfer the supernatant to the same 0.5 mL tube. 10m

25 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. Most gel bands contain 2h

amount of protein that can be resuspended in 20 µL.

26 Perform desalting using C18 tips (BAF_Protocol_003):
– 10 µL tips for a small amount of proteins
– 100 µL for a higher amount of proteins.

1h