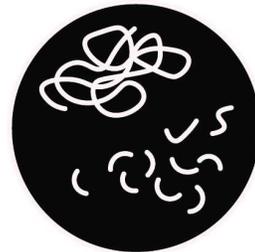


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## In-gel digestion protocol for protein identification

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Catarina Franco<sup>1</sup>

<sup>1</sup>MRC LMB



Catarina Franco

MRC LMB

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**Protocol status:** In development

**We are still developing and optimizing this protocol**

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**Keywords:** gel band analysis, mass spectrometry, protein identification, gel digestion protocol for protein identification, gel digestion protocol, gel digest, gel protocol, protein identification, protein, avoiding keratin contamination, sample for mass spec analysis, digestion, keratin contamination, mass spec analysis

## Abstract

This is the in-gel protocol we use in the facility and welcome you to use it to prepare your own in-gel digests. Once you complete the digestion you are ready to load Evotips with your sample for mass spec analysis. You can do this manually or with the help of a pipetting robot. Please see "Warning" section for tips on avoiding keratin contamination of your in-gel digests.

## Protocol materials

 Acetonitrile LC-MS grade B&J Brand **VWR International (Avantor) Catalog #BJLC015-2.5**

 Ammonium bicarbonate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A6141**

 Formic acid, LC-MS grade **Thermo Fisher Scientific Catalog #28905**

 Promega trypsin **Promega Catalog #V5113**

 DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D0632**

 Iodoacetamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #I1149-5G**

## Troubleshooting

## Safety warnings

-  Dust will be your worst enemy when trying to identify a protein from a gel. Most dust components are proteinaceous in nature (e.g., skin cells, jumper fibers). If your gel sits on the bench uncovered for long or you use tools that have been left sitting on the bench with no cover, rest assured we will mostly see keratin instead of your critical proteins. To limit keratin/dust contamination use clean surfaces: e.g., cut the band with a new/clean scalpel and always use gloves.

## Before start

Please make sure you have a clean surface and use clean tools to process your gels. This will avoid the dreaded keratin contamination.

## Band excision from an SDS-PAGE gel

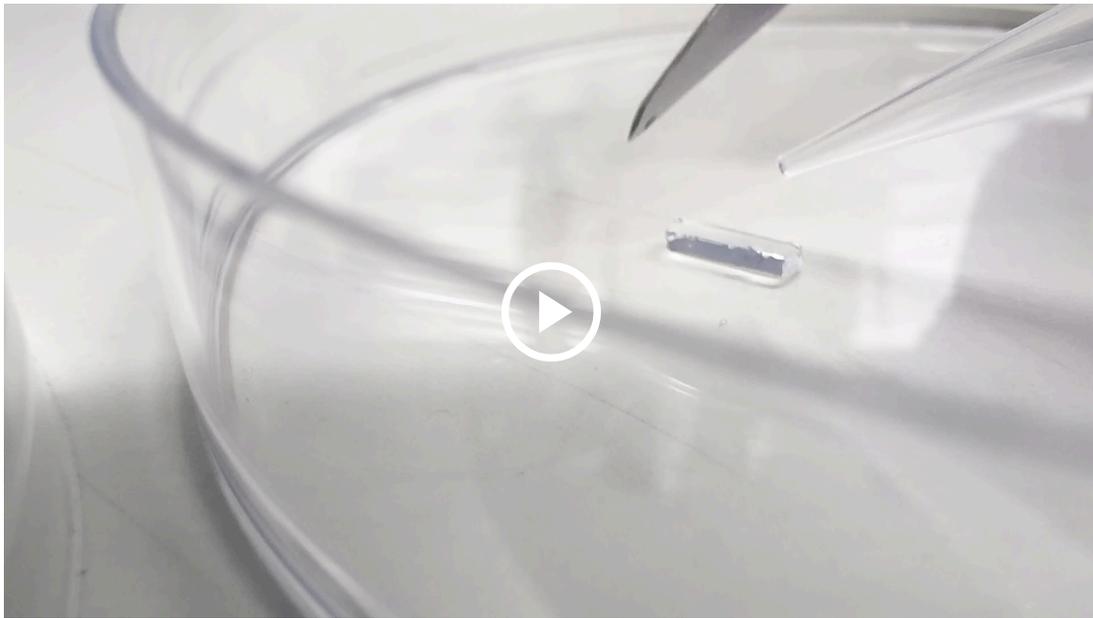
10m

- 1 Cut either a small (1mm<sup>3</sup>) plug of a stained protein band or excise an entire band and slice this into smaller sections, place material into a 1.5ml tube containing approximately

10m

 200  $\mu$ L 25mM AMBIC .

### Note



## Gel band destaining

- 2 Prepare two wash solutions:

**Solution A:** 2 parts of 25mM ammonium bicarbonate (AMBIC) (made up in HPLC water) mixed with 1 part acetonitrile

**Solution B:** 25mM ammonium bicarbonate (AMBIC) (made up in HPLC water)

 Ammonium bicarbonate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A6141**

Acetonitrile LC-MS grade B&J Brand **VWR International**  
 (Avantor) Catalog #BJLC015-2.5

- 3 Wash the band with  500  $\mu\text{L}$  of **solution A**. Incubate the gel plug for 15min at 37°C with gently agitation  800 rpm, 37°C, 00:15:00

15m

Note

If the band is extremely stained you can increase the volume of Solution A/B to approximately  1000  $\mu\text{L}$  .

Note

You can use an Eppendorf Thermomixer for the incubation steps at 37C or in alternative you can just shake at room temperature for  00:20:00

Equipment

<b>Eppendorf Thermomixer C Model 5382</b>	NAME
Thermomixer C	TYPE
Eppendorf	BRAND
5382000023	SKU

- 4 Remove and discard solution A from the tube containing the gel band.

- 5 Wash the band with  $500\ \mu\text{L}$  of **solution B**: 25mM ammonium bicarbonate (AMBIC) (made up in HPLC water). Incubate the gel plug for 15min at 37°C.

15m

↻ 800 rpm, 37°C, 00:15:00

- 6 Remove and discard solution B from the tube containing the gel band.

- 7 Repeat steps 2-7 until the gel band is fully destained.

#### Expected result



Destained gel band shown on the right.

It is really important that the gel bands are fully destained before progressing to the next step. This is usually attained by the end of two cycles of solution A/B washes. Once your gel is transparent it is ready for the next step - reduction and alkylation of cysteines.

## Reduction and alkylation of cysteines

1h

- 8 Wash the gel piece with  $500\ \mu\text{L}$  acetonitrile ↻ 800 rpm, 22°C, 00:15:00 .

- 9 Remove and discard the acetonitrile. Your gel band should have a whitish appearance when dry.

### Expected result



Dehydrated gel band.

The gel should look white (dehydrated) as seen in the above picture. If the band is still transparent then repeat steps 8-9 until fully dehydrated.

- 10 Prepare a solution of 1.5mg/mL of DTT [M] 10 millimolar (mM) and 10mg/mL of iodoacetamide [M] 60 millimolar (mM) in 25mM AMBIC.

 DTT Merck MilliporeSigma (Sigma-Aldrich) Catalog #D0632

 Iodoacetamide Merck MilliporeSigma (Sigma-Aldrich) Catalog #I1149-5G

- 11 Add enough volume of the DTT solution to fully cover the gel band.

#### Note

You usually need around 50uL of volume to fully cover the gel band.

- 12 Incubate for 60min at 60C  800 rpm, 60°C, 01:00:00

1h



13 Let the tube cool down and spin down to return liquid to bottom of tube.

14 Aspirate the liquid around the gel plug using a pipette and discard.

15 Add enough volume of the iodoacetamide solution to fully cover the gel band.

#### Note

You only need around 50uL of iodoacetamide per band.

16 Incubate at room temperature, IN THE DARK for 45min  00:45:00 in the dark

45m

17 Wash the gel piece with  200  $\mu$ L 25mM AMBIC  800 rpm, 22°C, 00:10:00

10m

18 Remove and discard the supernatant.

19 Wash the gel piece with  500  $\mu$ L acetonitrile  800 rpm, 22°C, 00:15:00 .

15m

20 Remove and discard the acetonitrile. Your gel band should have a whitish appearance when dry.

### Expected result



Dehydrated gel band.

The gel should look white (dehydrated) as seen in the above picture. If the band is still transparent then repeat steps 17-18 until fully dehydrated.

## Overnight digestion with trypsin

16h

- 21 Prepare a trypsin solution of 6ng/uL using 25mM AMBIC and add enough to fully cover the dehydrated gel piece.

 Promega trypsin **Promega Catalog #V5113**

- 22 Add 25uL of the above trypsin solution and place the gel piece in the fridge for 10min so it fully rehydrates in the trypsin solution.

- 23 Incubate overnight at 37C  800 rpm, 37°C, 16:00:00

16h

## Extract tryptic peptides

30m

- 24 Quickly spin down the digest to bottom of tube. Add formic acid (FA, 10% v/v) to attain a final concentration of 1% (v/v). Aspirate and RETAIN solution, which will contain the

30m

peptides. To extract more peptides, soak the gel piece in 10uL of a solution containing water:Acetonitrile:formic acid (50:49:1) at 37°C for  00:30:00 . Pool the peptides.

 Formic acid, LC-MS grade **Thermo Fisher Scientific Catalog #28905**

25 Dry the extracted peptides to completion using a speedvac.