WHOLE-GEL IN-GEL DIGESTION (IGD)

Description

Proteins contained in a polyacrylamide gel slice are enzymatically digested into peptide fragments with trypsin, which are then extracted from the gel slice, and concentrated for further mass spectrometric (MS) analysis.

Principle

Polyacrylamide gel electrophoresis is a powerful tool whereby protein mixtures can be cleaned up and separated. Apart from separating away high molecular weight/neutral contaminants, separating the protein mixture into gel sections with a limited (smaller) number of different proteins reduces sample complexity, which enhances identification through MS.

In-gel digestion procedures involve enzymatically cutting up gel-contained proteins into peptides. This conversion into peptides has several advantages. First, it facilitates extraction from the polyacrylamide matrix. Second, it generates molecules with a size that is more compatible with present-day MS. Third, it produces peptides with a predicted C-terminal end (lysine or arginine; trypsin cuts C-terminal from these residues), which highly reduces the complexity of searching databases with theoretical mass spectra. Fourth, it may also enable protein isoform discrimination, and/or identification of post-translationally modified proteins. Fifth, it (optionally) allows liquid chromatography-based separation of extracted peptides before mass spectrometry, to further reduce sample complexity and increase the number of identified peptides and proteins.

In whole-gel IGD, polyacrylamide gels as a whole are washed and brought to tryptic digestion conditions (pH conditioning and removal, a.o., of most Coomassie and SDS). Cysteine residues that may be present in particular proteins in the gel are reduced and blocked by alkylation, in order to break and prevent reformation of disulfide bridges (residual protein folding will reduce accessibility of trypsin target sites). Then, specific regions of the gel are cut out, cut into pieces, and processed separately for tryptic digestion. The denatured proteins contained in the washed gel slices are subjected to digestion *in situ* ("in-gel") with a sequence-specific protease (usually trypsin, but other enzymes may also be used) to generate peptide fragments. The peptides are subsequently extracted from the acrylamide matrix, and extracts are concentrated in a centrifugal evaporator for subsequent MS identification/quantification purposes.

Samples

Polyacrylamide gels as a whole are processed in the initial steps of the protocol. The samples are run only into the running gel for blob gel processing, or the full length for gel-based fractionation (Figure 1). Then, slices are cut from processed gels in a laminar flow cabinet, while wearing gloves and disposable plastic arm sleeves to prevent contamination by keratins, from, hair, skin particles, wool sweaters, or dust. Each gel slice serves as a sample for tryptic digestion and MS analysis.

When gels are to be used for IGD procedures, try to minimise keratin contamination already during gel processing (staining/destaining, etc.).

Gel pieces (cubes) should not be made much smaller than 1 mm^3 (clogging of pipette tips and risk of transfer to the LC column, which will also be clogged), nor should they be much larger (inefficient exchange of solutions). See figure 2.

Equipment

- Gel scanner
- Rocking platform
- Water bath
- Laminar flow cabinet
- Vortex mixer for multiple 1.5-ml tubes
- Microcentrifuge
- Centrifugal evaporator (SpeedVac)
- Thermoblock for controlled temperature incubations

Materials

- Clean gel-size, closable container (e.g., blue pipette tip box with inner grid removed)
  This container should be protein free, so not used for, e.g., westernblot blocking!
- Disposable graduated pipettes, e.g. Corning Costar Stripette serological pipettes (cat# 4051, cat# 4101, or cat# 4251 for 5-ml, 10-ml, or 25-ml pipettes, respectively)
- Clean glass plate
- Razor blades
- Saran wrap/household foil
- Aluminum foil
- Pipette tips
- Plastic tubes
  These should not give off polymers, and be low adsorbent (minimising peptide loss). Polymers are detrimental to subsequent MS analysis. E.g., 50-ml tubes of Greiner Bio-One (cat# 210261) and 1.5-ml Safe-Lock microtubes of Eppendorf (cat# 0030 120.086) can be safely used.

Reagents

Commercial Products

- Sequencing-grade trypsin, e.g. Sequencing Grade Modified Trypsin supplied by Promega: freeze-dried aliquots of 20 μg/vial (cat# V5111).
- (Optional) Trypsin resuspension buffer, e.g. Trypsin Resuspension Buffer (50 mM acetic acid) supplied by Promega (cat# V542A). This can, however, easily be made in the lab.

Reagents

Solids

Trivial Name	IUPAC Name	Chemical Formula	Mr	CAS Number
Ammonium bicarbonate	Ammonium bicarbonate	NH_4HCO_3	79.06	[1066-33-7]
Dithiothreitol	(2S,3S)-1,4-Bis-sulfanylbutane-2,3-diol	$C_4H_{10}O_2S_2$	154.25	[3483-12-3]
Iodoacetamide	Iodoacetamide	C_2H_4INO	184.96	[144-48-9]

Liquids

Trivial Name	**IUPAC Name**	**Chemical Formula**	**Mr**	**CAS Number**
MilliQ water ("double-distilled ~")	Oxidane	H_2O	18.02	[7732-18-5]
Acetonitrile	Ethanenitrile	C_2H_3N	41.05	[75-05-8]
Acetic acid	Ethanoic acid	$C_2H_4O_2$	60.05	[64-19-7]
Formic acid	Methanoic acid	CH_2O_2	46.03	[64-18-6]

Solutions

Abbreviation	**Chemical Composition**
ABC	50 mM ammonium bicarbonate
ABC/ACN	50 mM ammonium bicarbonate / 50% acetonitrile
DTT	10 mM dithiothreitol in 50 mM ammonium bicarbonate
IAA	54 mM iodoacetamide in 50 mM ammonium bicarbonate
FA	1% formic acid
FA/ACN	5% formic acid / 50% acetonitrile
10xTrypsin	63 ng/ml Sequencing-grade trypsin in 50 mM acetic acid
Trypsin	6.3 ng/ml Sequencing-grade trypsin in 50 mM ammonium bicarbonate
Trypsin Resuspension Buffer	50 mM acetic acid

Preparation of reagents

Solutions should be prepared with keratin-free materials, and labelled with contents, name and date.
The EXAMPLE RECIPES below indicate recommended *amounts/volumes; alternatively, use equivalent ratios thereof, depending on the size/number of gel slices you have, and how much reagent you need.*

10xABC

0.5 M NH_4HCO_3 :

Weigh 1.0 gram NH_4HCO_3, transfer to a 50-ml Greiner tube, and dissolve in 25 ml MilliQ water to reach 40 mg/ml (0.5 M).

ABC

50 mM NH_4HCO_3 :

Dilute 5 ml 10xABC tenfold by adding MilliQ water to a final volume of 50 ml, giving a final concentration of 4 mg/ml (50 mM).

ABC/ACN

50 mM NH_4HCO_3 / 50% acetonitrile :

Dilute 5 ml 10xABC fivefold by adding MilliQ water to a volume of 25 ml, and add 25 ml acetonitrile to reach a final volume of 50 ml, and a final concentration of 4 mg/ml (50 mM).

DTT

10 mM DTT :

Beforehand, warm refrigerator-stored container with solid DTT to room temperature before opening!

Weigh 38-39 mg dithiothreitol in a 1.5-ml Eppendorf tube. From a 50-ml Greiner tube containing 25 ml ABC, use some ABC to dissolve the DTT, and return the solution to the Greiner tube, to obtain a 1.5 mg/ml DTT solution (1.54 mg/ml or 38.5 mg/25 ml= 10 mM).

IAA

54 mM IAA :

Beforehand, warm refrigerator-stored container with solid IAA to room temperature before opening!
Iodoacetamide is light sensitive, so always protect from light with, e.g., aluminum foil.

Weigh 250-260 mg iodoacetamide, and dissolve in 25 ml ABC in a 50-ml Greiner tube to obtain a 10 mg/ml IAA solution (10.17 mg/ml or 254 mg/25ml = 55 mM).

1% FA *(pre-made)*

1% FA :

Dilute 500 µl formic acid into some MilliQ water in a 50-ml Greiner tube, and bring final volume to 50 ml with MilliQ water.

> *To pipette formic acid, use a **glass** pasteur pipette attached to a micropipette via a plastic tip.*

FA solution/ACN *(pre-made)*

5% FA / 50% acetonitrile :

Dilute 2.5 ml formic acid into some MilliQ water in a 50-ml Greiner tube, bring volume to 25 ml with MilliQ water, and then add 25 ml acetonitrile to obtain a final volume of 50 ml.

> *To pipette formic acid, use a **glass** pasteur pipette attached to a micropipette via a plastic tip.*

Trypsin

6.3 ng/ml trypsin in 50 mM NH_4HCO_3 :

Make just before use, ON ICE

If the complete contents of a vial is going to be used:

Directly dissolve trypsin in ABC.

Dissolve freeze-dried contents (20 µg) of one vial Sequencing Grade Modified Trypsin (Promega) in 3200 µl ice-cold ABC.

If only part of the contents of a 20-µg trypsin vial is going to be used:

Make a 10X stock of trypsin by dissolving the freeze-dried contents (20 µg) of one vial in 320 µL trypsin resuspension buffer (cat# V542A, tubes with pink caps) and dilute the needed amount 10X in ABC. Label the leftover vial and store at -20 °C.

Method

GENERAL

During all steps up till the first peptide extraction step with trypsin-inhibiting FA, extreme care should be taken to work keratin free:

- Wear a lab coat, disposable sleeves, and clean gloves at all times, and don't wear a wool sweater.
- Work as much as possible in a cleaned laminar flow cabin, e.g., when handling opened containers/tubes during pipetting, and when processing gels/gel slices.
- Remove hair/skin/dust particles from pipettes by cleaning briefly with wet lint-free tissue.
- Keep reagents (solids, solvents, and solutions) as well as sample tubes keratin free by staying away with your head/arms from the opening of tubes/containers at all times.

Method

PROTOCOL in-gel digestion (IGD)

The following protocol was devised for the use of Greiner pipette tip boxes (tip grid removed) as a gel container; when using a different container, adapt volume of solutions added to the container accordingly.

WASHING/EQUILIBRATION OF GEL

1. Start with a Coomassie-stained, MQ-washed gel. Scan a picture of the gel. **Clean the scanner** beforehand *and* afterwards with 70% ethanol.
2. Transfer the gel to a keratin-free, gel-size container with **25 ml ABC**. Rock closed container on a platform for **10 min at RT**, and remove solution with a 25-ml pipette.
3. Add **25 ml ABC/ACN** to the container with the ge**l**. Rock closed container on a platform for **10 min at RT**, and remove solution with a 25-ml pipette. Discard in organic waste bin.
4. Repeat step 3 once.

REDUCTION AND ALKYLATION OF PROTEINS

5. Add **25 ml DTT** to the container with the gel. Wrap closed container in Saran wrap/household foil, incubate for **1 h at 56°C** in a water bath, and remove solution with a 25-ml pipette.
6. Add **25 ml IAA** to the container with the gel. Wrap closed container in aluminum foil (protect IAA solution from light), rock on platform for **45 min at RT**, and remove solution with a 25-ml pipette.

WASHING AND CUTTING OF GEL SLICES

7. Add **25 ml ABC** to the container with the gel. Rock closed container on a platform for **10 min at RT**, and remove solution with a 25-ml pipette
8. Add **25 ml ABC/ACN** to the container with the gel. Rock closed container on a platform for **10 min at RT**, and remove solution with a 25-ml pipette. Discard in organic waste bin.
9. Add **25 ml ABC** to the container with the gel. Rock closed container for **10 min at RT**.

   **** If the Coomassie staining is very faint, incubate for a shorter time to be able to see the protein in the next steps.*
10. In a laminar flow cabinet, place the gel on a clean, keratin-free glass plate (white paper underneath the glass plate will enhance visualisation).
11. "Slice-and-Dice": cut protein-containing gel lanes into slices, cut (dice) the slices into 1-mm^3 cubes, and transfer cubes to labelled 1.5 mL Eppendorf microcentrifuge tubes.

    **** For blob-gel: cut out the protein blob including part of the stacking region.*

    **** For gels that have been run for the full length: Cut the lanes into sections no bigger than 1/3rd of the lane, first cutting horizontally (leaving the marker intact to keep the gel together), then cut each lane, one section at a time.*

    **** Do NOT cut gel slices into TOO small pieces (< 1mm^3), as these will clog the pipette tips. Do also not make them much larger, as this will reduce efficiency of liquid exchange.*

    **** Do NOT cut through fat bands (like albumin bands), but retain them in one slice per lane.*
12. To all tubes with diced gel slices, add **300 µl ABC/ACN**, incubate at 400 rpm shaking for **10 min at RT**, and remove solution by pipetting.
13. Dry gel pieces in a centrifugal evaporator (SpeedVac) for **30 min** at **60 °C** or until no excess liquid is visible (Figure 3).

IN-GEL TRYPTIC DIGESTION

14. *Just before use* (while drying gel pieces), prepare cold **Trypsin** solution on ice.
15. To the dried gel pieces, add sufficient **Trypsin** solution to completely cover the gel pieces (usually 150-250µL, take into account reswelling of pieces), and incubate at 400 rpm shaking for 10 min.
16. Add sufficient **ABC** (150-300 µL) to completely cover the gel pieces, and incubate O/N at 25 °C in a thermoblock. Alternatively, this step can be performed for 4-6 h at 37 °C.

EXTRACTION OF TRYPTIC PEPTIDES

17. Add **100-150 µl 1% FA** to the tubes with gel pieces/ABC, and vortex GENTLY for 15 min.

    *** *Do NOT vortex too vigorously (setting ~ 5-6 on a Vortex-Genie 2, Scientific Industries), as gel pieces will fragment by vigorous vortexing.*

    *** *Trypsin is inhibited by low pH, so now the keratin danger is over (as keratins are not cut into tryptic peptides anymore), and working in a laminar flow cabinet is no longer necessary*.

18. Collect the peptide extract by pipetting, and transfer it to a fresh, clearly labelled **low-binding** 1.5-ml Eppendorf tube ("extract collection tube").
19. Add **100-150 µl FA/ACN** to the tubes with gel pieces, and vortex GENTLY for 15 min.
20. Collect the peptide extract by pipetting, and transfer it to the extract collection tube already containing the first (FA) extract.
21. Repeat steps 19-20, ending with a peptide extract resulting from 1 FA and 2 FA/ACN extractions.

STORAGE/PROCESSING OF EXTRACTS

22. Store extracts "as is" at -20 °C in box labelled with:
    - Project name + OPL code (if available)
    - Date
    - Name + contact details

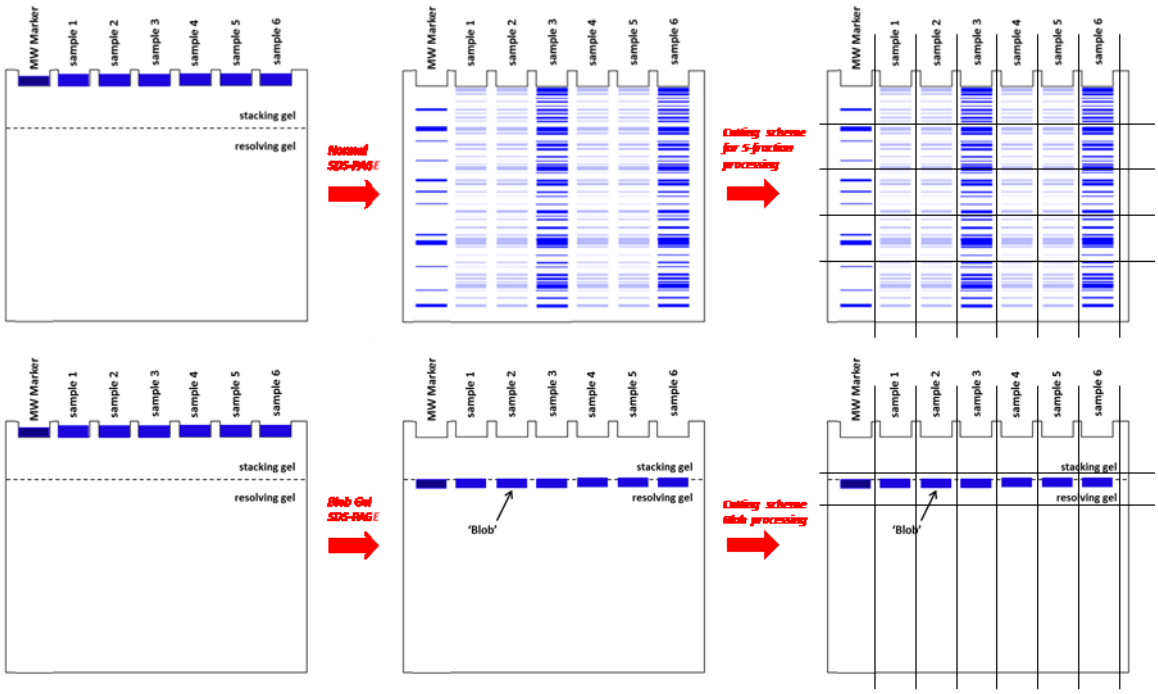

Figure 1: Full-length gel vs. Blob gel

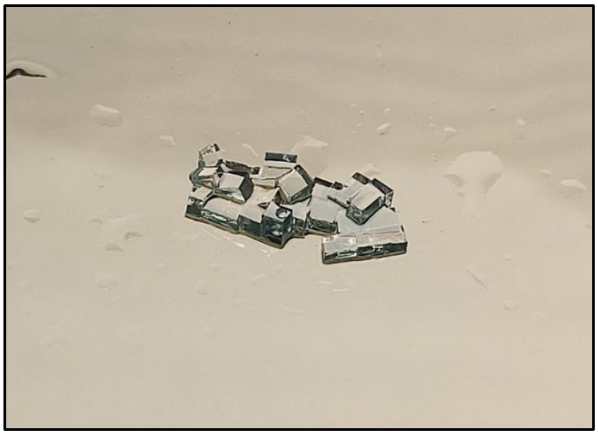

Figure 2: Cut gel pieces

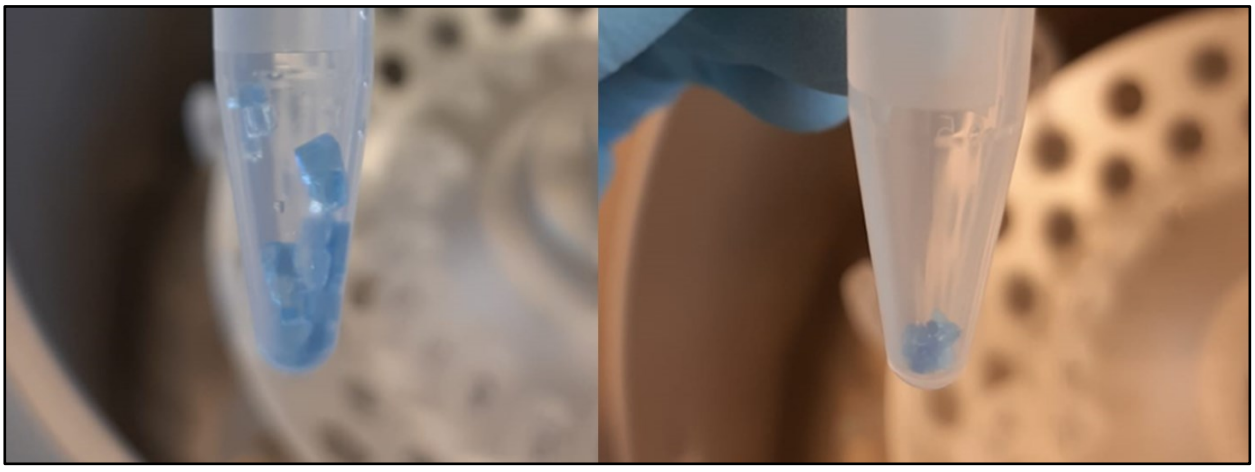

Figure 3: Gel pieces before and after drying in speedvac