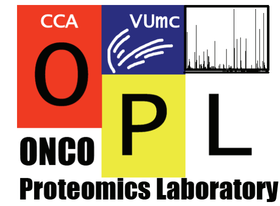
WHOLE-GEL IN-GEL DIGESTION (IGD)



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.

The "Whole-gel" designation refers to the pretreatment phase of the protocol: not until the stage of tryptic digestion is the gel cut into separate slices containing specific regions of the gel. Thus, all (initial) washing, reduction, and alkylation steps are performed with the gel as a whole; only then are small slices cut from the gel for tryptic digestion (in contrast, the "Slice-By-Slice" IGD procedure immediately starts out with small gel slices already cut out of a gel). Therefore, the whole-gel procedure is useful for the processing of large gel areas which are to be cut into many separate slices. When a small number of gel slices are to be analysed, or when gel slices are derived from small parts of many different gels, use the "Slice-By-Slice IGD" protocol.

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 IDG, 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. 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 then 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).

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 savely 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.

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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

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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).

FA

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.*

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FA/ACN

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 Resuspension Buffer

50 mM HAc :

Dilute 144 μl acetic acid into 50 ml MilliQ water (glacial acetic acid is 17.5 M at room temperature). If available, Promega's resuspension buffer (cat# V542A) may also be used.

10xTrypsin Stock

63 ng/ml trypsin in 50 mM HAc :

ON ICE

Dissolve freeze-dried contents (20 μg) of one vial Sequencing Grade Modified Trypsin (Promega) in 320 μl Trypsin Resuspension Buffer, aliquot, and freeze aliquots for future experiments at -20 ^{o}C.

Trypsin

6.3 ng/ml trypsin in 50 mM NH_4HCO_3 :

Make just before use, ON ICE

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

Dilute sufficient 10xTrypsin Stock tenfold with ABC.

E.g., dilute 100 μl 10xTrypsin with 900 μl ice-cold ABC to obtain 1 ml trypsin solution.

Alternatively, 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 in 3200 μl ice-cold ABC.

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 clean(ed) 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.

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Method

DETAILED PROTOCOL WHOLE-GEL IGD

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

WASHING/EQUILIBRATION OF GEL

1. 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.
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.
9. Repeat step 7.
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 Eppendorf microcentrifuge tubes.

    *** *Using the gel picture scanned earlier as a guide, cut slices from the gel with a clean razor blade, such as exemplified in Supplementary Figure 1 (Gel Slicing for IGD).Often, a whole gel lane containing a cell/tissue lysate is cut into 10 slices (for less complex samples one can reduce the number of slices, i.e. generate larger slices, when adapting solution volumes accordingly).*

    *** *Do NOT cut gel slices into TOO small pieces (< $1mm^3$), as these will clog both pipette tips and LC columns! 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**, vortex GENTLY for **10 min at RT**, and remove solution by pipetting.
13. Dry gel pieces in a centrifugal evaporator (SpeedVac) for **10 min** at **50 °C**.

    *** *For the Jouan RC10-09 evaporator in CCA 1-53, first start centrifugation, then apply vacuum by turning valve 90^0 clockwise (arrow pointing upward); to stop, first release vacuum by returning valve to original position (arrow pointing to speedvac-cold trap combination), then stop centrifugation.*

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 50-100 µl, take into account reswelling of pieces), and vortex GENTLY for 10 min.
16. Briefly spin down in a microcentrifuge, remove all solution by pipetting (make sure all liquid outside the gel pieces is removed to prevent a background of trypsin autodigestion in solution).
17. Add sufficient **ABC** to completely cover the gel pieces, and incubate O/N at 25 ^{o}C in a thermoblock. Alternatively, this step can be performed for 4-6 h at 37 ^{o}C.

EXTRACTION OF TRYPTIC PEPTIDES

18. Add **100-150 µl 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.*

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

STORAGE/PROCESSING OF EXTRACTS

If MS analysis is not being done on the same day:

23. Store extracts "as is" at -20 ^{0}C in a clearly labelled box with name/project code, title, and date.

Whenever MS analysis is being done:

24. Concentrate peptide extracts, and concomitantly remove acetonitrile, by reducing extract volume to **≤ 50 µl** in a centrifugal evaporator (SpeedVac) at **50 ^{o}C**.

    *** *Do NOT allow ALL liquid to evaporate, as this may result in peptide loss due to adsorption of peptides to the plastic tube*

25. Bring extract volumes to exactly **50 µl** with FA for comparitive (quantitative) nanoLC-MS/MS analyses.
26. Transfer concentrated extracts to labelled autosampler vials for the nanoLC.
    Label each vials with sample ID (e.g., "A1"), initials, project code, (short title), and date.

<End of Detailed Protocol>

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Method

CONCISE PROTOCOL

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

WHOLE-GEL IGD: DAY1

WASHING/EQUILIBRATION OF GEL

1. Scan a picture of the gel.
2. Transfer gel to a container with **25 ml ABC**. Rock for **10 min at RT**, remove solution.
3. Add **25 ml ABC/ACN**. Rock for **10 min at RT**, remove solution.
4. Repeat step 3 once.

REDUCTION AND ALKYLATION OF PROTEINS

5. Add **25 ml DTT**. Wrap container in Saran wrap/household foil, incubate for **1 h at 56 °C** in a water bath, remove solution.
6. Add **25 ml IAA**. Wrap container in aluminum foil, rock for **45 min at RT**, remove solution.

WASHING AND CUTTING OF GEL SLICES

7. Add **25 ml ABC**. Rock for **10 min at RT**, remove solution.
8. Add **25 ml ABC/ACN**. Rock for **10 min at RT**, remove solution.
9. Repeat step 7.
10. In a laminar flow cabinet, place gel on a clean glass plate.
11. Cut gel lanes into slices, dice slices into 1-mm^3 cubes, and transfer cubes to labelled microtubes.
12. Add **300 µl ABC/ACN**, vortex GENTLY for **10 min at RT**, remove solution.
13. Dry gel pieces in a centrifugal evaporator for **10 min** at **50 °C**.

IN-GEL TRYPTIC DIGESTION

14. *Just before use*, prepare cold **Trypsin** solution on ice.
15. Add sufficient **Trypsin** solution to completely cover gel pieces, vortex GENTLY for 10 min.
16. Briefly spin down, remove all liquid.
17. Add sufficient **ABC** to completely cover gel pieces, incubate O/N at 25 °C (or 4-6 h at 37 °C).

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WHOLE-GEL IGD: DAY2

EXTRACTION OF TRYPTIC PEPTIDES

18. Add **100-150 µl FA** to the digestion tubes, vortex GENTLY for 15 min.
19. Transfer peptide extracts to labelled 1.5-ml Eppendorf tubes ("extract collection tubes").
20. Add **100-150 µl FA/ACN** to the tubes with gel pieces, vortex GENTLY for 15 min.
21. Transfer peptide extracts to the "extract collection tubes".
22. Repeat steps 20-21.

STORAGE/PROCESSING OF EXTRACTS

If MS analysis is not being done on the same day:

23. Store extracts at -20 ^{0}C in a clearly labelled box with name/project code, title, and date.

Whenever MS analysis is being done:

24. Concentrate extracts to ≤ **50 µl** in a centrifugal evaporator **at 50 ^{o}C**.
25. Bring extract volume to exactly **50 µl** with FA for comparitive (quantitative) purposes.
26. Transfer concentrated extracts to labelled autosampler vials for the nanoLC.
    Label each vial with sample ID (e.g., "A1"), initials, project code, (short title), and date.

<End of Concise Protocol>