# SLICE-BY-SLICE 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 "Slice-by-slice" designation refers to the fact that the procedure immediately starts with small slices that are cut from a gel (as opposed to the "Whole-gel" procedure, where the initial stages of the procedure are performed with the gel as a whole). The Slice-by-slice in-gel digestion (IGD) procedure is useful when a small number of gel slices (e.g., from distinct regions of a gel) are to be analysed. When a large number of gel slices from the same gel are to be analysed, use the "Whole-gel in-gel digestion" 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 Slice-by-slice IGD, protein-containing slices are cut from polyacrylamide gels, and then washed and brought to tryptic digestion conditions (pH conditioning and removal, a.o., of SDS and most Coomassie). Cysteine residues that may be present in particular proteins in the gel are reduced and blocked by alkylation, in order to break disulfide bridges and prevent their reformation (residual protein folding will reduce accessibility of trypsin target sites).Then, 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

Gel slices are cut from polyacrylamide gels in a laminar flow cabinet, while wearing gloves and disposable plastic arm sleeves to prevent contamination by keratins present in hair, skin particles, wool sweaters, and dust. Each gel slice is cut ("diced") into ~ 1 $mm^3$ cubes, and transferred to a pre-labelled Eppendorf tube.

*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).*

*Microcentrifuge tubes should be the ones provided by Eppendorf, since for these tubes it is known that they do not give off high amounts of polymers during the procedure, and are made of low-adsorbing polypropylene (minimising peptide loss). Polymers are detrimental to subsequent MS analysis.*

If not immediately proceeding with the IGD workflow, the gel pieces can be stored at 4 $^{o}$C in water for short-term, or 0.1% acetic acid for long-term storage.

# Equipment

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

# Materials

- Clean glass plate
- Razor blades
- 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.

# 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.*

**2xABC**

**100 mM $NH_4HCO_3$ :**

Weigh 0.4 gram $NH_4HCO_3$, transfer to a 50-ml Greiner tube, dissolve in 50 ml MilliQ water to reach 8 mg/ml (100 mM), and divide solution over two 50-ml Greiner tubes (25 ml/tube).

**ABC**

**50 mM $NH_4HCO_3$ :**

Dilute contents of one tube containing 25 ml 2xABC twofold by adding 25 ml 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 contents of the other tube containing 25 ml 2xABC twofold by adding 25 ml acetonitrile to a final volume of 50 ml, giving 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 15 mg dithiothreitol in a 1.5-ml Eppendorf tube, and dissolve in 1 ml ABC to obtain a 10x concentrated solution (15 mg/ml ~ 100 mM).

**To obtain 10 mM DTT** : dilute the above 100 mM DTT solution tenfold in ABC.

E.g., add 100 µl 100 mM DTT to 900 µl ABC to obtain 1 ml 10 mM DTT.

**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.*

**** For 10-20 samples(depending on size of gel slices):*

Weigh 10 mg iodoacetamide in an Eppendorf tube, and dissolve in 1 ml ABC to obtain a 10 mg/ml solution (54 mM) of IAA.

**** For more samples/bigger gel slices:*

Weigh 100 mg iodoacetamide in an Eppendorf tube, and dissolve in 1 ml ABC to obtain a concentrated stock solution of 10xIAA (0.54 M).

**To obtain 54 mM IAA from 10x stock** : dilute the above 10xIAA solution tenfold in ABC.

E.g., add 500 µl 0.54 M IAA to 4500 µl ABC to obtain 5 ml 54 mM IAA.

**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.*

**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.

**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 °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.

# Method

## DETAILED PROTOCOL SLICE-BY-SLICE IGD

### CUTTING OF GEL PIECES

1. Scan a picture of the gel. **Clean the scanner** beforehand *and* afterwards with 70% ethanol.
2. In a laminar flow cabinet, place the gel on a clean, keratin-free glass plate (white paper underneath the glass plate will enhance visualisation).
3. "Slice-and-Dice": Cut gel slices from protein-containing areas of interest, cut (dice) the slices into 1-$mm^3$ cubes, and transfer cubes to labelled microcentrifuge tubes.

   *** *Using the gel picture scanned earlier as a guide, cut the region of interest from the gel with a clean razor blade, cut the slice in cubes of ~ 1 $mm^3$,and transfer gel pieces (cubes) to an Eppendorf tube labelled with the slice ID (see Supplementary Figure 1-Gel Slicing for IGD).*

   *** *Do NOT cut gel slices into TOO small pieces (< 1$mm^3$), 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.*

4. I you want to stop here, add ~ 200 µl MilliQ water (short-term storage) or 0.1% acetic acid (long-term storage) to the tubes with gel cubes, and store all tubes at 4 °C. *In that case, when you want to resume the procedure, start with step 5.* Otherwise, proceed with step 6.

### WASHING/EQUILIBRATION OF GEL PIECES

5. If processing previously stored tubes with gel cubes, remove all storage liquid.
6. Add **400 µl ABC**, vortex GENTLY for **10 min at RT**, and remove all solution by pipetting.

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

7. Add **400 µl ABC/ACN**, vortex GENTLY for **10 min at RT**, and remove all solution by pipetting.
8. Repeat step 7 once.

### REDUCTION AND ALKYLATION OF PROTEINS (*for 2D gel spots, skip & proceed with step 16*)

9. Add sufficient **DTT** to completely cover the gel pieces (50-100 µl), and incubate for **1 h at 56 °C** in a thermoblock.
10. Briefly spin down in a microcentrifuge, remove all solution by pipetting.
11. Add sufficient **IAA** to completely cover the gel pieces (same volume as step 9), cover tubes with aluminum foil, and incubate in the dark for **45 min at RT** with occasional vortexing.
12. Briefly spin down, remove all solution by pipetting.

### WASHING AND DRYING OF GEL PIECES

13. Add **400 µl ABC**, vortex GENTLY for **10 min at RT**, and remove all solution by pipetting.
14. Add **400 µl ABC/ACN**, vortex GENTLY for **10 min at RT**, and remove all solution by pipetting.
15. Repeat step 14 once.
16. 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

17. *Just before use* (while drying gel pieces), prepare cold **Trypsin** solution on ice.
18. 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.
19. 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).
20. 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

21. 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*.

22. Collect the peptide extract by pipetting, and transfer it to a fresh, labelled 1.5-ml Eppendorf tube ("extract collection tube").
23. Add **100-150 µl FA/ACN** to the tubes with gel pieces, and vortex GENTLY for 15 min.
24. Collect the peptide extract by pipetting, and transfer it to the extract collection tube already containing the first (FA) extract.
25. Repeat steps 23-24, 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*:

26. 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*:

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

    A) 50 µl (for nanoLC-MS/MS analyses) or

    B) 10-20 µl (for ZipTipping prior to MALDI-TOF/TOF analyses).

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

28. For

    A) NanoLC-MS/MS analyses, concentrated extracts are transferred to autosampler vials for the nanoLC.

    B) MALDI-TOF/TOF analyses, concentrated extracts are desalted using C18 Zip Tips, mixed with MALDI matrix, and spotted onto a MALDI target plate

***<End of Detailed Protocol>***

# Method

## CONCISE PROTOCOL

## SLICE-BY-SLICE IGD: DAY1

### CUTTING OF GEL PIECES

1. Scan a picture of the gel.
2. In a laminar flow cabinet, place the gel on a keratin-free glass plate.
3. Cut slices from the gel, dice into 1-$mm^3$ cubes, and transfer cubes to labelled tubes.
4. STOPPING: add ~ 200 µl MilliQ water (short-term storage) or 0.1% acetic acid (long-term storage), and store tubes at 4 °C. *When resuming the procedure, start with step 5.*
   PROCEEDING: skip step 5, and go on with step 6.

### WASHING/EQUILIBRATION OF GEL PIECES

5. If processing previously stored tubes with gel cubes, remove all storage liquid.
6. Add **400 µl ABC**, vortex GENTLY for **10 min at RT**, remove all solution.
7. Add **400 µl ABC/ACN**, vortex GENTLY for **10 min at RT**, remove all solution.
8. Repeat step 7 once.

### REDUCTION AND ALKYLATION OF PROTEINS (*for 2D gel spots, skip & proceed with step 16*)

9. Add sufficient **DTT** to cover gel pieces (50-100 µl), incubate for **1 h at 56 °C**.
10. Briefly spin down, remove all solution.
11. Add sufficient **IAA** to cover gel pieces, incubate for **45 min at RT in the dark**, vortex occasionally.
12. Briefly spin down, remove all solution by pipetting.

### WASHING AND DRYING OF GEL PIECES

13. Add **400 µl ABC**, vortex GENTLY for **10 min at RT**, remove all solution.
14. Add **400 µl ABC/ACN**, vortex GENTLY for **10 min at RT**, remove all solution.
15. Repeat step 14 once.
16. Dry gel pieces in a centrifugal evaporator for **10 min at 50 °C**.

### IN-GEL TRYPTIC DIGESTION

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

## EXTRACTION OF TRYPTIC PEPTIDES

21. Add **100-150 µl FA** to the digestion tubes, vortex GENTLY for 15 min.
22. Transfer extracts to fresh, labelled 1.5-ml Eppendorf tubes ("extract collection tubes").
23. Add **100-150 µl FA/ACN** to the tubes with gel pieces, vortex GENTLY for 15 min.
24. Transfer extracts to the extract collection tubes.
25. Repeat steps 23-24.

## STORAGE/PROCESSING OF EXTRACTS

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

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

*Whenever MS analysis is being done*:

27. Concentrate peptide extracts in a centrifugal evaporator at **50 °C** to:
    A) 50 µl (for nanoLC-MS/MS analyses) or
    B) 10-20 µl (for MALDI-TOF/TOF analyses).
28. For
    A) NanoLCMS/MS analyses, transfer concentrated extracts to autosampler vials for nanoLC.
    B) MALDI-TOF/TOF analyses, desalt concentrated extracts using C18 Zip Tips, mix with MALDI matrix, and spotted onto a MALDI target plate

***<End of Concise Protocol>***