SOP	department of protein science (prot)		
TITLE	**In-Gel-Digest**		
CATEGORY	**Digestion**	*AUTHOR*	**Core Facility Proteomics**
VERSION #	**4.1**	*DATE*	**1.12.2010**

REAGENTS

- Use HPLC water (Merck) for the preparation of all solutions.

- Silver destain solution: Dissolve 0,1 g / 10 ml potassium ferricyanide (Potassium hexacyanoferrate(III); Merck, 1.06509.0100) to gain a 30 mM solution. Dissolve 0,25 g / 10 ml sodium thiosulfate (Sodium thiosulfate pentahydrate; Merck, 1.06509.0100) to gain a 100 mM solution. Destain with a 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate

- 5mM DTT Solution (1,4-Dithiothreitol; Merck; 1 .11 474.0025). Always prepare a fresh solution or prepare 1 M stocks and freeze 50 µl aliqots at -20 °C

- 25 mM IAA Solution (2-Iodoacteamid; Merck; 8.04744.0025). Always prepare a fresh solution and store at RT in total darkness until usage.

- ABC Solution (Ammonium Bicarbonate; SIGMA; A61 41 -25G):
    - 50 mM solution: Dilute 199mg ABC in 50 ml HPLC water.

Please note: Always prepare a fresh solution. ABC is an unstable buffer and becomes acidic over time, degrading into ammoniac, CO_2 and H_2O!

- ACN Dilutions (acetonitrile hypergrade for LC/MS; Merck; 1 .00029.1 000). Do not use plastic reservoirs for acetonitrile containing solutions!

- TFA Solutions (Trifluoracetic Acid, protein sequencing grade; AB Sciex (Applied Biosystems); 400003; 40 ml). Do not use plastic reservoirs for acetonitrile and TFA containing solutions and try not to use plastic tips as well!

- Trypsin, sequencing grade (Sigma, proteomics grade, T6567-5x20ug).

Dissolve lyophilised Trypsin (20 µg) by applying 40 µl H_2O, 1 mM; HCl. → This can be divided into 5 µl (2,5 µg) aliquots with a concentration of 0,5 µg / µl and stored at -20 °C.

- Before usage dilute one Trypsin aliquot (2,5 µg) with 245 µl **50 mM** ABC solution for LC-MS/MS approaches.
- For a MALDI run dilute the same amount Trypsin in 245 µl **25 mM** ABC (higher salt concentrations (> 30 mM) will affect the MALDI ionization process).
- This results in a final concentration of 0.01 µg/µl of Trypsin.

PROCEDURE

Some general Do's and Don'ts for gel handling:

☐ Wear gloves at all times during sample handling!!!!!

☐ Cut gel bands on a clean bench and a clean glass plate using a clean (=new) scalpel knife

☐ Use clean containers with lids to stain and store gels

☐ Please take care that no contamination (skin, hair etc.) can enter the open tubes

☐ For all steps that include a solution the gel pieces/ spots have to be completely covered!

1st Day:

Digestion

- Cut out gel pieces with a scalpel
- Transfer pieces into tubes (standard Eppendorf) or a 96 well plate (we recommend VWR: 732-2702, Lid: 734-2185; no polymers are extracted)
- Destaining
  - for silver stained
    - De-stain with a 1:1 solution of 30mM potassium ferricyanide (0.1 g / 10 ml) and 100mM sodium thiosulfate (0.25 g / 10 ml) (~5 minutes, until destained)
    - Wash 3x with 200 µl H_2O (1x short, 2 x 5 minutes)

  - for Coomassie stained (H_2O and acetonitrile steps should be alternating)
    - Wash 1x 10 minutes with 200 µl 60 % acetonitrile
    - Wash 1x 10 minutes with 200 µl H_2O
    - During this step the gel pieces are dehydrated which removes the stain. The gel pieces should shrink and become white. The

alternating H_2O/organic step is necessary for the dehydration to be effective.

- Please note: You have to repeat the wash-steps until the spots are completely destained!

1. Protein reduction and alkylation (this is not necessary for 2D-Spots)
   - Add 100 µl 5 mM DTT, incubate 15 minutes 60 °C (prepare 1M stocks and freeze at -20 °C)
   - Remove DTT solution
   - Add 100 µl freshly prepared 25 mM iodacetamide (0.046 g / 10 ml), incubate 15 minutes at RT in the dark.
   - Removal of DTT and iodacetamid from gel pieces:
   - Wash 1 x 5 minutes with 100 µl H_2O
   - Wash 1 x 10 minutes with 100 µl 100 % acetonitrile
   - Wash 1 x 10 minutes with 100 µl 50 mM ammoniumbicarbonate
   - Wash 1 x 10 minutes with 100 µl 60 % acetonitrile
   - Wash 1 x 10 minutes with 100 µl 100 % acetonitrile

2. Trypsin digestion
   - Prepare the Trypsin stock with 1 mM HCL as described in the manual
   - Overlay the gel pieces with 0,01 µg / µl Trypsin diluted in 50 mM ammoniumbicarbonat, for MALDI analysis use 25 mM ammoniumbicarbonat instead
   - After 10 Min add only as much of the 25 mM ammoniumbicarbonat solution as is necessary to cover the gel pieces completely with liquid (for MALDI analysis use 10 mM ammoniumbicarbonat!).
   - Please note: It is important to cover the spots completely, because otherwise the spots run dry overnight. But do not use too much liquid because the solution for direct MALDI analysis will be otherwise too diluted!
   - Digest overnight at 37 °C

2nd Day:

Elution

- Add 1-2 µl 0.5 % TFA, shake 15 minutes (to stop the trypsin activity) This step is not mandatory when samples are eluted subsequently with acetonitrile and 0.1 % TFA.
- For direct MALDI analysis take 1µl supernatant and spot it on the MALDI target. If your spots are very small the protein concentration may be not enough for a successful protein ID and you have to elute them.
- For elution transfer supernatant to a tube and combine it with the solutions from the next two elution steps.
- Add enough volume of 60 % acetonitrile / 0.1 %TFA in order to cover the gel pieces completely and shake for 15 minutes.
- Add 100 % acetonitrile / 0.1 % TFA and shake again for 15 minutes and combine with the other fractions. As a result the gel pieces should shrink and become white.
- Dry samples in the speed vac to complete dryness (Be careful not to prolong this step, the peptides stick to the plastic of the tube the longer you speed-vac them!)
- Re-dissolve in 2 % ACN / 0.5 % TFA (This step is performed by the core facility staff immediately before the analysis!!)