IN-GEL ALKYLATION AND DIGESTION WITH MODIFIED TRYPSIN (mTr)

1. Excise the stained protein gel band or spot. Cut the gel piece into ½ x ½ mm cubes and put them in a microcentrifuge tube. If silver stained, destaining is not compulsory, go to step 3.

2. Coomassie stained gel pieces need to be destained: Rinse the gel pieces three times in 200 µl 0.25 M NH_4HCO_3/ACN (1:1) for 20 min at 37 ºC (until pieces become white).

3. Dehydrate 2x the gel pieces by adding 200 µl ACN. Dry in vacuum centrifuge for 5 min.

4. Rehydrate the gel pieces in 100 µl 20 mM DTT in 0.1 M NH_4HCO_3 for 30 min at 56 ºC.

5. Remove excess liquid and dehydrate the pieces with ACN as above (3).

6. Add 100 µl 55 mM iodoacetamide in 0.1M NH_4HCO_3. Incubate for 15 min at RT, in the dark.

7. Remove excess liquid and wash the pieces with 200 µl 0.1 M NH_4HCO_3 and dehydrate with ACN as above (3).

8. Add mTr solution (as follows) and allow absorbing for 30 min at RT. Then add digestion buffer to cover the gel pieces and incubate o/n at 37 ºC.

	Tr solution	Digestion buffer	mg of mTr/sample
Coomassie stained 1D band	10 µl	5-15 µl	0,5 µg
Silver stained 1D band	10 µl 1:2 dilution	5-15 µl	0,25 µg
Silver stained 2D spot	5 µl 1:5 or 1:10 dilution	2-10 µl	0,025-0,05 µg

The peptides are recovered as follows:

9. Transfer supernatant to another clean tube
10. Add 10 µl of 25 mM NH_4HCO_3 to the gel pieces and incubate 15 min at RT. Recover the supernatant

11. Incubate the gel pieces twice in 10 µl of 5 % formic acid for 15 min at RT. All supernatants should be combined. No drying is needed prior to Millipore µC18 Zip Tip-purification.

12. Perform the ZipTiping according to the protocol. Elute with 1.5µl 50 % ACN/0.1 % TFA; let it dry. Repeat this step once or twice. Make sure that there is enough eluting liquid in the tip!!!

13. Finally add 1.5µl CHCA matrix solution. Let it dry again and ready for MALDI analysis.

Solutions:
0.1M NH_4HCO_3: Dilute 1 M (7.9 g/100 ml) 1+9 with water
20 mM DTT: Dilute 1 M (0.3897 g/2.5 ml) 2 µl DTT + 98 µl NH_4HCO_3
55 mM iodoacetamide: Dilute 1M (1.0096 g/ 5.5 ml) 5.5 µl IAA + 94.5 µl NH_4HCO_3
mTr: modified trypsin 0.05µg/µl in water
CHCA matrix solution: ~10mg/ml in 0.1% TFA/33% ACN = small amount in tube in ~200ul