In-gel digest (Coomassie stained) with trypsin

Adapted from:
Shevchenko, A., Wilm, M., Vorm, O and Mann, M. (1996). Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. *Anal. Chem.* 68, 850-858.

A. Excision of protein bands from polyacrylamide gels

A1. Wash the gel with water (2 changes, 10 minutes each)
A2. Excise the spots of interest from the gel. Cut the excised piece into 1 mm^2-cubes.
A3. Transfer the gel cube to a 0.5 ml Eppendoff tube.

B. Washing of gel pieces

B1. Wash the gel particles with 50 µl wash buffer (10 mM NH_4HCO_3, 50% ACN) for 15 min.
B2. Remove all remaining liquid and add 100 µl ACN to cover the gel particles. The gels shrink and stick together.
B3. Remove the ACN and rehydrate the gel pieces with 25 µl 20 mM NH_4HCO_3 for 5 min.
B4. Add 25 µl ACN and mix well.
B5. Remove all liquid after 15 min of incubation. (if Coomassie blue is not removed, repeat step B2 to B5)
B6. Add 100 µl ACN to cover the gel particles for 5 min.
B7. After the gel pieces have shrunk, remove the ACN.
B8. Dry down gel particles in a vacuum centrifuge or air dry.

C. Reduction and alkylation

C1. Add 50 µl reduction solution (10 mM DTT/ 10 mM NH_4HCO_3 (freshly prepared).
C2. Incubate for 15 min at 56°C, then chill tubes to room temperature.
C3. Remove liquid and replace it quickly by roughly the same volume as above of freshly prepared 50 µl alkylation solution (55 mM IAA/ 10 mM NH_4HCO_3). * light sensitive!!
C4. Incubate for 20 min at room temperature in the dark.
C5. Remove alkylation solution.
C6. Wash the gel with 50 µl wash buffer for 15 min, 1 or 2 times.
C7. Add 100 µl ACN to cover the gel particles.
C8. After the gel pieces have shrunk, remove the ACN.
C9. Dry down the gel particles in a vacuum centrifuge or air dry.

D. In-gel digestion

D1. Add 3 µl freshly prepared enzyme solution (20 ng/µl of trypsin, in 10 mM NH_4HCO_3) to cover the gel.
D2. Incubate at 4°C for 60 min.
D3. Add 3 µl 10 mM NH_4HCO_3 to keep the gel wet overnight, but avoid excess liquid.
D4. incubate at 37°C overnight or at 56°C for 60min.

E. Extraction of peptides

E1. Make sure that about 3 µl of liquid is in the tube.
E2. Add 2 µl of 50% ACN with 1% TFA or formic acid.
E3. Sonify 10 min sonication, recover the supernatant.
E4. Repeat the step E2 and E3.
E5. pool the supernatant.

Mass spectrometric peptide analysis:
Preferably, use an AnchorChip target for sample preparation.
If purification is necessary, perform microcolumn purification.

General remarks size of gel pieces:
Cut as close to protein band as possible to reduce the amount of "background" gel.
Avoid to use gel pieces much bigger than 1 mm × 1 mm.
Excise a gel piece of roughly the same size from a non-protein containing region of the gel for use as a control.

Solvent amounts:
Unless otherwise specified all the solvent volumes used in the washing steps should roughly equal twice the gel volume.

Solvent purity:
All solvent should be as pure as possible. Make sure that the bottles remain close when not in use.
Lab material purity:
Scalpel, pipette tips and tubes should be clean. Use original Eppendorff bubes.

Contamination by keratin:
Keratin contamination should be avoided. Avoid keratin to fall on the gel, in bottles or on pipette tips. It might help to use clean gloves.

Prepare the trypsin solution:
Sequencing grade, modified trypsin in recommended. The solid powder is solved in 1 mM hydrochloride acid (for Roche Diagnostic, Mannheim trypsin) or 50 mM acetic acid (for Promega trypsin) giving a concentration of 100 ng/µl, store 10 µl aliquots at -20°C.