In-gel digestion protocol

Reagent preparation:

* All solutions should be made with HPLC-grade water, acetnitrile.
And all reagents should be the highest grade.

1. Wash solution: 25 mM ammonium bicarbonate

1 M ammonium bicarbonate	75 ul
water	3 ml

2. Reducing solution

1 M DTT (771 mg/ 5 ml water)	10 ul
1 M Ammonium bicarbonate	25 ul
Water	965 ul

(prepare when in use, do not stock)

3. Alkylation solution: 55 mM iodoacetamide, 25 mM Am-bicarbonate)

Iodoacetamide	10 mg

dissolve in 1 ml wash solution

4. Dehydration solution

100 % Acetonitrile (ACN)	2 ml
1 M am-bicarbonate	100 ul
water	1.9 ml

5. Trypsin solution

Dissolve trypsin GOLD (100 ug) in 50 mM Acetic acid 1ml.
Make aliquots and stock in -80°C.(we have the stock)
mix:

Trypsin solution (100 ug/ml)	20 ul
50 mM Am-bicarbonate	180 ul

In-gel digestion procedure:

1. Wash the gel (SDS-PAGE) with milli-Q several times

2. Pick (Cut out) the protein band.

3. Slice the gel into small pieces
(the cutter knife should be wiped with 100% methanol)

4. De-staining
(a) in case of silver-stain
Add 400 ul milli-Q, shake for 10 min, and remove solution, repeat 2 times
(b) in case of CBB-stain
Add 400 ul destaining buffer (50%ACN, 25 mM Ammonium bicarbonate)
Shake for 10 min
Remove the solution
Repeat until the gel shows no blue color
If difficult to destain, ACN concentration can be reduced to 30%

5. Dehydration
Add 200 ul ACN
Centrifuge (appropriate)
Remove supernatant
Vacuum dry in a desiccator

6. Reducing alkylation
Add 100 ul reducing reagent (10 mM DTT, 25 mM Ammonium bicarbonate)
Incubate at 55°C for 1 h.
Remove supernatant
Add 100 ul 25 mM ammonium bicarbonate
Shake at RT for 10 min (to remove DTT)
Remove supernatant by centrifuge
Add 100 ul of Alkylating reagent (55 mM Iodoacetamide, 25 mM Am-bicarbonate)
Shake at RT for 45 min in dark
Remove supernatant
Add 100 ul wash solution (25 mM Am-bicarbonate)
Shake at RT for 10 min

*If you do these steps (dehydration and reducing alkylation), cysteines are "carbamidemethyl"ated, so you have to select it as a "fixed modification" when you try MASCOT search (later).

7. In-gel Digestion
    Add 400 ul dehydration solution (50% ACN, 25 mM Am-bicarbonate)
    Shake at RT for 10 min
    Repeat 2-3 times
    Add 200 ul ACN
    Centrifuge (appropriate)
    Vacuum dry in a desiccator
    Add trypsin solution (10 ng/ul, 25 mM Am-bicarbonate) 15-30 ul
    Incubate at 37°C O/N

8. Extraction
    Add 50%ACN / 5%TFA 100 ul, vortex
    Centrifuge at 5000 rpm
    Transfer supernatant into new tube
    Repeat again and combine the supernatant together
    Add 200 ul ACN, centrifuge, and combine supernatant together
    Vacuum dry the obtained supernatant (it should be 400 ul) until it become about 40 ul.

---> proceed to MS/MS analysis