

# Method for in-gel *S*-pyridylethylation of gel resolved proteins

## 1. Prepare the following stock solutions:

0.2M Tris (pH 8.4)

2.422 g Trizma (Sigma, Cat.# T-1503)  
100 ml deionized water

0.1M EDTA

3.722 g EDTA (Ajax, Cat.# 28417)  
100 ml deionized water

Reduction Buffer (10mM DTT, 2mM EDTA, 0.2M Tris pH 8.4)

0.154 g dithiothreitol (DTT, Cleland's reagent) (Calbiochem, Cat.# 233153)  
98 ml 0.2M Tris (pH 8.4)  
2 ml 0.1M EDTA

## 2. Reduction

### For whole gels:

1. Ensure that the gel has been appropriately stained and destained.
2. Wash the intact gel extensively in large volumes of deionized water, typically 400-500ml x 3 changes over approximately 1.5 hours.  
(This step is included to remove the acetic acid from the destain protocol which would otherwise adversely affect the pH of the reduction.)
3. Transfer the intact gel to a clean container.
4. Immerse the gel in 50ml of reduction buffer (volume depends on the size of both the gel and the container).
5. Incubate the gel at 40°C for 2 hours.

### For individual gel bands:

1. Ensure that the gel was appropriately stained and destained.
2. Place excised gel piece in a clean eppendorf (1.5ml polypropylene tube).
3. Wash the excised gel piece extensively with deionized water, 1ml x3 changes over approximately 1.5 hours.  
or  
Completely dehydrate the gel by centrifugal lyophilization.  
(This step is included to remove the acetic acid from the destain protocol which would otherwise adversely affect the pH of the reduction.)
4. Remove deionized water from eppendorf.
5. Add enough reduction buffer to completely cover gel piece (typically 100-200µl).  
(Dehydrated gel pieces will swell as they absorb the reduction buffer. Ensure there is enough buffer to accommodate this.)
6. Incubate the gel at 40°C for 1 hour.

### **3. S-Pyridylethylation**

#### **For whole gels:**

1. Add 4-vinylpyridine (2% v/v) to container holding the intact gel in reduction buffer. (1ml / 50ml)
2. Incubate the gel in the dark at room temperature for 1 hour.
3. Halt alkylation with the addition of excess  $\beta$ -mercaptoethanol (2% v/v).
4. Wash the gel extensively in large volumes of deionized water, typically 400-500ml x 3 changes over approximately 1.5 hours. (This step is included to remove  $\beta$ -mercaptoethanol which would otherwise interfere with proteolysis.)
5. If protein spots are no longer visible repeat stain and destain procedure.

#### **For individual gel bands:**

1. Add 4-vinylpyridine (2% v/v) to the eppendorf containing the gel piece in reduction buffer.
2. Incubate the gel piece in the dark at room temperature for 1 hour.
3. Halt alkylation with the addition of excess  $\beta$ -mercaptoethanol (2% v/v).
4. Wash the gel piece extensively in large volumes of deionized water, typically 1ml x 3 changes over approximately 1.5 hours. (This step is included to remove  $\beta$ -mercaptoethanol which would otherwise interfere with proteolysis.)

# Method for in-gel proteolysis of gel resolved proteins

## 1. Prepare the following stock solutions:

### For in-situ trypsin cleavage:

0.2M  $\text{NH}_4\text{HCO}_3$  (Ammonium Bicarbonate)  
1.581 g  $\text{NH}_4\text{HCO}_3$  (BDH, Cat.# 10302 5E)  
in 100 ml deionized water

10mM  $\text{CaCl}_2$  (Calcium Chloride)  
0.147 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (BDH, Cat.# 10070)  
in 100 ml deionized water

Digestion Buffer (0.2M  $\text{NH}_4\text{HCO}_3$ , 0.5mM  $\text{CaCl}_2$ )  
95 ml 0.2M  $\text{NH}_4\text{HCO}_3$   
5 ml 10mM  $\text{CaCl}_2$

0.1M  $\text{NH}_4\text{HCO}_3$  / 50%  $\text{CH}_3\text{CN}$  (Acetonitrile)  
50 ml 0.2M  $\text{NH}_4\text{HCO}_3$   
50 ml  $\text{CH}_3\text{CN}$

### For in-situ *Achromobacter lyticus* protease I (Lys-C) cleavage:

Digestion Buffer (0.1M Tris-HCl pH 9.3)  
1.211 g Trizma (Sigma, Cat.# T-1503)  
in 100 ml deionized  $\text{H}_2\text{O}$  (pH with HCl)

0.05M Tris-HCl, pH 9.3 / 50%  $\text{CH}_3\text{CN}$   
50 ml 0.1M Tris-HCl pH 9.3  
50 ml  $\text{CH}_3\text{CN}$

### Extraction Buffers:

1% TFA (Trifluoroacetic Acid)  
99 ml deionized  $\text{H}_2\text{O}$   
1 ml TFA (Pierce, Cat.# 28901)

0.1% TFA / 60%  $\text{CH}_3\text{CN}$   
60 ml  $\text{CH}_3\text{CN}$   
39.9 ml deionized  $\text{H}_2\text{O}$   
0.10 ml TFA

## 2. Excise stained gel band:

1. Remove CBR-250 by washing with 1 ml of either 0.1M  $\text{NH}_4\text{HCO}_3$  / 50%  $\text{CH}_3\text{CN}$  (for trypsin) or 0.05M Tris-HCl, pH 9.3 / 50%  $\text{CH}_3\text{CN}$  (for Lys-C) x2 for 30 min at 30°C.
2. Dry the gel piece completely by centrifugal lyophilization. (The dried gel piece should not stick to the walls of the eppendorf tube.)

### **3. In-gel digestion:**

1. Rehydrate gel band by adding 5  $\mu$ l digest buffer containing 0.5  $\mu$ g enzyme directly onto the gel piece.
2. Wait until solution has been absorbed by the gel piece. (~5-10 min)
3. Repeat steps 1-2 until desired enzyme to substrate ratio is achieved.
4. Add 200  $\mu$ l of digest buffer to the eppendorf containing the gel piece.
5. Incubate at appropriate conditions. Typically 37°C for ~16 h (overnight).

### **4. Peptide extraction:**

1. Carefully remove digest/incubation buffer and collect in a separate, clean eppendorf. (The digest/incubation buffer contains >80% of extractable peptides resulting from in gel proteolysis.)
2. Add 200  $\mu$ l of 1% TFA to the eppendorf containing the gel piece.
3. Place in a sonicating bath for 30 min.
4. Carefully remove 1% TFA and add it to eppendorf containing the digest/incubation buffer.
5. Add 200  $\mu$ l of 0.1% TFA / 60% CH<sub>3</sub>CN to the eppendorf containing the gel piece.
6. Place in a sonicating bath for 30 min.
7. Carefully remove 0.1% TFA / 60% CH<sub>3</sub>CN and add it to the eppendorf containing the digest/incubation and 1% TFA extraction buffer.
8. Reduce the volume of the pooled extracts by centrifugal lyophilization.  
(Do not dry pooled extracts completely otherwise sample loss may result. The objective of this step is to remove CH<sub>3</sub>CN from the pooled extracts and reduce the extracts to a volume suitable for dilution and loading onto capillary HPLC systems.)