

## **Methylation-sensitive Southern blotting**

To determine the methylation level of the *CpoI* restriction site in the proximal (p13-E11 probe) and all (D4Z4 probe) D4Z4 repeat units on chromosome 4

### **1. Digestion with *BlnI***

<i>BlnI</i> (10 u/μl):	1.5 μl
Buffer K (10x):	6.0 μl
DNA (5 μg) + H <sub>2</sub> O:	<u>52.5 μl</u>
	60.0 μl

- Incubate overnight at 37 °C

### **2. Precipitation**

- 1/10 x 60 μl = 6 μl NaAc (3M, pH 5.3)
- 2.5 x 60 μl = 150 μl 100% EtOH
- Precipitate for 30 minutes at -80 °C
- Centrifuge: 20-30 minutes, 14000 rpm, 4 °C
- Remove supernatant from pellet
- Wash pellet with 200 μl 70% EtOH
- Centrifuge: 10-15 minutes, 14000 rpm, 4 °C
- Remove supernatant from pellet -> dry pellet (≤ 5 minutes)
- Dissolve pellet in 30 μl Tris-HCl (10mM, pH 7.5)
- Incubate for 20 minutes at 65 °C

### **3. Digestion with *Eco91I* + *CpoI***

Buffer O (10x):	6.0 μl
<i>Eco91I</i> (10 u/μ):	1.5 μl
<i>CpoI</i> (10 u/μl):	3.0 μl
<i>BlnI</i> digested sample:	30.0 μl
H <sub>2</sub> O:	<u>19.5 μl</u>
	60.0 μl

- Incubate overnight at 37 °C

### **4. Samples on gel**

- 0.8% TAE gel (300 ml 1x TAE buffer + 2.4 gram agarose)
- Load 60 μl sample + 12 μl blue LB (5x)
- Load 10 μl 1kb marker
- Run 15 minutes at 35 V
- Run for 7-8 hours at 80 V

### **5. Blotting**

- Make picture of gel
- Shake gel 2x 20-30 minutes in blot buffer
- Needed for blotting:
  - 3x Whatman filter size gel
  - 1x Whatman filter around glass plate
  - 1x Hybond-XL membrane size gel
  - tray for buffer

- paper towels
- glass plate
- Saran wrap
- Blot overnight
- Neutralize membrane in neutralization buffer (> 10 minutes)
- Dry membrane (> 1 hour)
- Crosslink membrane (1200)

## 6. Prehybridization

- Shake membrane for a minimum of 30 minutes at 65 °C
  - 35 ml NaPi/SDS/PEG hybridization buffer (65 °C)
  - 350 µl fish sperm (10'/95 °C)

## 7. Probe labelling (Megaprime DNA labelling kit)

- 11.5 µl p13-E11 DNA (15 ng) + H<sub>2</sub>O
- 2.0 µl primer
- Incubate for 5 minutes at 95 °C
- Incubate for 5 minutes at room temperature
- 4.0 µl buffer + 1.0 µl enzyme + 1.5 µl <sup>32</sup>P-dCTP
- Incubate for a minimum of 20 minutes at 37 °C
- Add 15 µl stopmix to probe
- Add 300 µl TE<sup>-4</sup>
- Add marker probe (prepared from 3 µl standard DNA in kit)
- Probe for 10 minutes at 95 °C
- Add probe to membrane
- Incubate overnight at 65 °C (shake in waterbath)

## 8. Wash membrane

- Wash membrane 3x 5 minutes in 2x SSC/0.1% SDS
- Wash membrane 1x 15 minutes in 1x SSC/0.1% SDS
- Wash membrane 1x 15 minutes in 0.3x SSC/0.1% SDS
- Expose membrane overnight to phosphorimager screen
- Scan phosphorimager screen (Storm)
- Analyze blots with Image Quant software (methylation of first repeat unit)

## 9. Strip membrane

- Shake membrane in just boiled 0.1x SSC/0.1% SDS for 30 minutes at room temperature (2x)

## 10. Prehybridization

- Rotate membrane for a minimum of 30 minutes at 62 °C
  - 15 ml formamide hybridization buffer (62 °C)
  - 10 ml formamide (deionize before use)
  - 250 µl fish sperm (10'/95 °C)

## 11. Probe labelling (Megaprime DNA labelling kit)

- 11.5 µl D4Z4 DNA (15 ng) + H<sub>2</sub>O

- 2.0 µl primer
- Incubate for 5 minutes at 95 °C
- Incubate for 5 minutes at room temperature
- 4.0 µl buffer + 1.0 µl enzyme + 1.5 µl <sup>32</sup>P-dCTP
- Incubate for a minimum of 20 minutes at 37 °C
- Add 15 µl stopmix to probe
- Add 300 µl TE<sup>-4</sup>
- Add marker probe (prepared from 3 µl standard DNA in kit)
- Probe for 10 minutes at 95 °C
- Add probe to membrane
- Incubate overnight at 62 °C (rotate in incubator)

## 12. Wash membrane

- Wash membrane 2x 10 minutes in 2x SSC/0.05% SDS
- Wash membrane 2x 10 minutes in 0.1x SSC/0.1% SDS
- Expose membrane overnight to phosphorimager screen
- Scan phosphorimager screen (Storm)
- Analyze blots with Image Quant software (methylation of all repeat units)

### Blot buffer

- 175 gram NaCl
- 80 gram NaOH
- Add demi-H<sub>2</sub>O to 5 liter



### NaPi/SDS/PEG hybridization buffer

- Prepare 0.5 liter NaPi buffer
  - dissolve 44.45 gram Na<sub>2</sub>HPO<sub>4</sub> in 400 ml MQ-H<sub>2</sub>O
  - pH 7.2 with 85% H<sub>3</sub>PO<sub>4</sub>
  - add MQ-H<sub>2</sub>O to 500 ml
- Add to 0.5 liter NaPi buffer
  - 100 ml 5M NaCl
  - 4 ml 0.5M EDTA (pH 8.0)
  - 140 gram SDS (=7% final)
  - 200 gram PEG-6000
  - add MQ-H<sub>2</sub>O to 2 liter

### Formamide hybridization buffer

- Prepare 0.5 liter NaPi buffer
  - dissolve 44.45 gram Na<sub>2</sub>HPO<sub>4</sub> in 400 ml MQ-H<sub>2</sub>O
  - pH 7.2 with 85% H<sub>3</sub>PO<sub>4</sub>
  - add MQ-H<sub>2</sub>O to 500 ml
- Add to 0.5 liter NaPi buffer
  - 100 ml 5M NaCl
  - 140 gram SDS (=7% final)
  - add MQ-H<sub>2</sub>O to 1 liter

### Stopmix

- 25 mM EDTA

- 2% blue dextran
- 0.2% phenol red

### **Neutralization buffer**

- 100 ml Tris (1M, pH 7.5)
- 50 ml 20x SSC
- 350 liter demi-H<sub>2</sub>O

### **Manufacturers**

*Bln*I: Takara

*Eco*91I: Fermentas

*Cpo*I: Fermentas

Buffer K: Takara

Buffer O: Fermentas

1kb marker: Fermentas

Whatman paper (GB003): Whatman

Hybond-XL: Amersham

Megaprime DNA labelling kit: Amersham

Formamide: J.T. Baker