

## Methylation-sensitive Southern blotting

To determine the methylation level of the *Bsa*AI and *Fse*I restriction sites in the proximal D4Z4 repeat unit on chromosome 4

### **1A. Digestion with *Bgl*III + *Bln*I + *Eco*RI (for *Bsa*AI digestion)**

<i>Bgl</i> III (10 u/μl):	1.2 μl
<i>Eco</i> RI (10 u/μl):	1.2 μl
<i>Bln</i> I (10 u/μl):	1.2 μl
Buffer H (10x):	6.0 μl
Spermidine (0.1 M):	4.0 μl
DNA (4 μg) + H <sub>2</sub> O:	<u>44.0 μl</u>
	57.6 μl

- Incubate overnight at 37 °C

### **1B. Digestion with *Bgl*III + *Bln*I + *Eco*RI (for *Fse*I digestion)**

<i>Bgl</i> III (10 u/μl):	1.5 μl
<i>Eco</i> RI (10 u/μl):	1.5 μl
<i>Bln</i> I (10 u/μl):	1.5 μl
Buffer H (10x):	6.0 μl
Spermidine (0.1 M):	4.0 μl
DNA (5 μg) + H <sub>2</sub> O:	<u>45.5 μl</u>
	60.0 μl

- Incubate overnight at 37 °C

### **2. Precipitation (only *Fse*I samples!!!)**

- 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

### **3A. Digestion with *Bsa*AI**

- Add 2.4 μl *Bsa*AI (5 u/μl)
- Incubate overnight at 37 °C

### **3B. Digestion with *Fse*I**

NEBuffer 4 (10x):	6.0 μl
BSA (100x):	0.6 μl
<i>Fse</i> I (5 u/μl):	6.0 μl
H <sub>2</sub> O:	<u>17.4 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 6-7 hours at 75 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)

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#### 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

### **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

### **Stopmix**

- 25 mM EDTA
- 2% blue dextran
- 0.2% phenol red

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### **Neutralization buffer**

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

### **Manufacturers**

*Bgl*III: Fermentas

*Bln*I: Takara

*Eco*RI: Fermentas

*Bsa*AI: New England Biolabs

*Fse*I: New England Biolabs (store at -80 °C!)

Buffer H: Roche -> alternatively, buffer Orange from Fermentas

Spermidine: Sigma Aldrich

NEBuffer 4: New England Biolabs

BSA: New England Biolabs

1kb marker: Fermentas

Whatman paper (GB003): Whatman

Hybond-XL: Amersham

Megaprime DNA labelling kit: Amersham