

# Chapter 2

## Materials and Methods

### 2.1 Materials

All buffers and solutions were made with deionised water and either filtered through sterile 0.2  $\mu\text{m}$  filters or autoclaved.

#### 2.1.1 Stock solutions

50 mg mL <sup>-1</sup> ampicillin	Frozen in aliquots and stored at -20°C
10% w/v APS	Stored in cold room (4°C) for up to one week
1 mg mL <sup>-1</sup> CaCl <sub>2</sub> .2H <sub>2</sub> O/ FeCl <sub>3</sub> (Ca <sup>2+</sup> / Fe <sup>3+</sup> )	Stored in cold room
1 $\mu\text{g mL}^{-1}$ CuSO <sub>4</sub> / MnCl <sub>2</sub> .4H <sub>2</sub> O/ ZnCl <sub>2</sub> / Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O (trace elements)	Stored in cold room
1M DTT	Frozen in aliquots and stored at -20°C
10mg mL <sup>-1</sup> EtBr	Light sensitive, stored in dark
4.9 M MgCl <sub>2</sub> (Sigma)	
3 M NaAc pH 5.2	
5 M NaOH	
1 M Tris-HCl pH 8.0	

#### 2.1.2 Bacterial cell culture solutions

Luria-Bertani (LB) medium	Tryptone	10 g
	Yeast extract	5 g
	NaCl	5 g
	dH <sub>2</sub> O	Up to 1 L
	pH to 7.2 with NaOH and autoclave.	

LB plates	Tryptone	10 g
	Yeast extract	5 g
	NaCl	5 g
	dH <sub>2</sub> O	Up to 1 L
	pH to 7.2 with NaOH	
	Agar	12.5 g
Autoclave same day. Antibiotics can be added when the agar has cooled to ~ 50°C and the resulting plates stored in dark at 4°C.		
2 × YT medium	Tryptone	16 g
	Yeast extract	10 g
	NaCl	5 g
	dH <sub>2</sub> O	Up to 1 L
	pH to 7.2 with NaOH and autoclave.	
FB1	RbCl	100 mM
	MnCl <sub>2</sub> .4H <sub>2</sub> O	50 mM
	KAc	30 mM
	CaCl <sub>2</sub> .2H <sub>2</sub> O	10 mM
	Glycerol	15% w/v
	pH to 6.8 with NaOH and filter sterilise.	
FB2	MOPS	10 mM
	RbCl	10 mM
	CaCl <sub>2</sub> .2H <sub>2</sub> O	75 mM
	Glycerol	15% w/v
	pH to 6.8 with NaOH and filter sterilise.	
SOC medium	Tryptone	20 g L <sup>-1</sup>
	Yeast extract	5 g L <sup>-1</sup>
	NaCl	0.5 g L <sup>-1</sup>
	KCl	25 mM

### 2.1.3 Gel solutions

5 × Orange G loading buffer	Glycerol	50% v/v
	Na <sub>2</sub> EDTA	100 mM
	Orange G	0.125% w/v
50 × TAE (National Diagnostics)	Tris-acetate	2 M
	Na <sub>2</sub> EDTA	100 mM
ProtoGel® (National Diagnostics)	Acrylamide	30% w/v
	Bis-acrylamide	0.8% w/v
Schägger PAGE gel buffer	Tris-HCl pH 8.45	3 M
	SDS	0.3% w/v
Schägger PAGE anode buffer	Tris-HCl pH 8.9	200 mM
Schägger PAGE cathode buffer	Tris-HCl	100 mM
	Tricine	100 mM
	SDS	0.1% w/v
2 × Schägger PAGE loading buffer	Tris-HCl pH 6.8	50 mM
	SDS	4% w/v
	Glycerol	12% w/v
	2-mercaptoethanol	2% v/v
	Coomassie Brilliant Blue G	0.01% w/v
Protein stain	Acetic acid	10% v/v
	Methanol	50% v/v
	Coomassie brilliant blue R-250	1 g L <sup>-1</sup>
Protein destain	Acetic acid	10% v/v
	Methanol	10% v/v

19:1 AccuGel™ (National Diagnostics)	Acrylamide	38% w/v
	Bis-acrylamide	2% w/v
29:1 AccuGel™ (National Diagnostics)	Acrylamide	38.62% w/v
	Bis-acrylamide	1.38% w/v
Formamide loading buffer	Formamide	10 mL
	Bromophenol blue	0.05% w/v
	Xylene cyanol FF	0.05% w/v
5 × Ficoll loading buffer	Tris-HCl pH 8.0	50 mM
	MgCl <sub>2</sub>	10 mM
	DTT	1 mM
	Ficoll	20 % w/v

#### 2.1.4 Protein purification solutions

##### *2.1.4.1 The S3 and S11 subunit*

Lysis buffer	sodium phosphate pH 8.0	50 mM
	NaCl	500 mM
Elution buffer	sodium phosphate pH 8.0	50 mM
	imidazole	500 mM
	NaCl	500 mM
Stripping buffer	Tris-HCl pH 8.0	50 mM
	NaCl	1 M
Nickel Ions	NiSO <sub>4</sub> ·6H <sub>2</sub> O	100 mM
Salt wash	NaCl	500 mM

#### 2.1.4.2 The M subunit

Lysis buffer	Tris-HCl pH 8.0	50 mM
	NaCl	100 mM
	sucrose	25 % w/v
	Na <sub>2</sub> EDTA	5 mM
	DTT	3 mM
IEX1	Tris-HCl pH 8.0	10 mM
	NaCl	100 mM
	Na <sub>2</sub> EDTA	1 mM
	DTT	1 mM
IEX2	Tris-HCl pH 8.0	10 mM
	NaCl	2 M
	Na <sub>2</sub> EDTA	1 mM
	DTT	1 mM
HIC1	ammonium sulphate	12 % w/v
	Tris-HCl pH 8.0	10 mM
	NaCl	100 mM
	Na <sub>2</sub> EDTA	1 mM
	DTT	1 mM
HIC2 / TNE	Tris-HCl pH 8.0	10 mM
	NaCl	100 mM
	Na <sub>2</sub> EDTA	1 mM
	DTT	1 mM

### 2.1.4.3 The R subunit

Lysis buffer	Tris-HCl pH 8.0	50 mM
	NaCl	100 mM
	Sucrose	25 % w/v
	Na <sub>2</sub> EDTA	5 mM
	DTT	3 mM
IEX3	Tris-HCl pH 8.0	10 mM
	NaCl	50 mM
	Na <sub>2</sub> EDTA	1 mM
IEX4	Tris-HCl pH 8.0	10 mM
	NaCl	100 mM
	Na <sub>2</sub> EDTA	1 mM

### 2.1.5 Miscellaneous solutions

Annealing buffer	Tris-HCl pH 8.2	10 mM
	NaCl	150 mM
	MgCl <sub>2</sub>	10 mM
10 × ThermoPol buffer (New England Biolabs)	KCl	100 mM
	Tris-HCl (pH 8.8 at 25°C)	200 mM
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	100 mM
	MgSO <sub>4</sub>	20 mM
	Triton X-100	1%
PCR master mix	10 × ThermoPol buffer	5 μL
	Template DNA	1 μL
	5' primer (50 μM)	0.5 μL
	3' primer (50 μM)	0.5 μL
	dATP (10 mM)	1 μL
	dCTP (10 mM)	1 μL
	dGTP (10 mM)	1 μL

	dTTP (10 mM)	1 $\mu$ L
	Vent <sub>R</sub> ® DNA polymerase (2 U/ $\mu$ L)	0.5 $\mu$ L
	dH <sub>2</sub> O	38.5 $\mu$ L
10 $\times$ T4 DNA ligase reaction buffer	Tris-HCl pH 7.5	500 mM
	MgCl <sub>2</sub>	100 mM
	DTT	100 mM
	ATP	10 mM
	BSA	250 $\mu$ g mL <sup>-1</sup>
10 $\times$ Polynucleotide Kinase (PNK) buffer (New England Biolabs)	Tris-HCl pH 7.6	700 mM
	MgCl <sub>2</sub>	100 mM
	DTT	50 mM

### 2.1.6 Suppliers

All chemicals were generally of AnalaR® or Molecular Biology grade and were purchased from Fisher, Merck or Sigma. Restriction enzymes and DNA markers were purchased from New England Biolabs (NEB). Protein markers were purchased from Invitrogen™. Expression vectors were purchased from Novagen. Oligonucleotides were purchased from Oswel or Invitrogen. Dialysis membranes were purchased from Spectrum®, and filter membranes and syringe filters were purchased from Whatman® and the Nalgene Company, respectively. Sample concentrators were purchased from Sartorius and Millipore.

## **2.2 Electrophoresis**

### 2.2.1 Agarose gels

DNA fragments were separated based on size using horizontal submarine agarose slab gels. The gel solution consisted of 1  $\times$  TAE and a percentage of agarose dependent on the size of the fragments to be separated. The mixture was melted in a microwave and allowed to cool to approximately 50°C before the addition of

4  $\mu$ L EtBr stock per 100 mL gel solution. The gel sledge was sealed at both ends with tape, the comb was inserted, and the gel was poured. The DNA samples to be analysed were mixed with a one-fifth volume of 5  $\times$  Orange G, mixed and loaded. The gel was run in 1  $\times$  TAE running buffer at 100 V. The DNA was then visualised using UV transillumination and depending upon the size of the fragment, the sizes were estimated by comparison with either 1 kbp (NEB) or 100 bp (NEB) DNA ladder (figure 2.1).

### 2.2.2 Tris-tricine polyacrylamide (Schägger) gels

Tris-tricine polyacrylamide gels (Schägger and von Jagow, 1987) can resolve low molecular weight proteins (10 kDa or less) such as S11, according to their molecular weight.

Glass plates, spacers and combs were first cleaned in dH<sub>2</sub>O and then degreased in EtOH. They were then assembled and clamped. The gels were then made according to table 2.1. After 6.6 mL resolving gel mixture was poured between the plates, a volume of water-saturated butanol was carefully added to ensure that there was an even interface between the resolving and stacking gels. When the resolving gel had set, the butanol was removed, the gel surface was rinsed with dH<sub>2</sub>O, and the stacking gel mixture was added. The comb was then placed into the gel. When the gel had set, the comb, spacer and clamps are removed and the gel was placed in the gel tank. Anode buffer was added to the outer section of the tank and cathode buffer was added to the inner section. The wells were then cleaned with cathode buffer.

Samples were prepared for electrophoresis by adding a one-half volume of 2  $\times$  Schägger PAGE loading buffer to them and heating at 95°C for 5 min. The samples were then centrifuged at 14,000 rpm for 1 min and loaded onto the gel. Each gel had 5  $\mu$ L Benchmark™ protein ladder (Invitrogen) loaded onto it (figure 2.1). The gels are run for 35 min at 60 V, followed by 150 V until the dye front reaches the bottom of the resolving gel. Following electrophoresis, the gel was removed and stained in stain solution for 1 h at room temperature and destained

Stock solutions	Stacking gel	Resolving gel	
		10%	12%
Protogel	405 $\mu$ L	2.5 mL	3 mL
Tris-HCl pH 8.45	775 $\mu$ L	2.5 mL	2.5 mL
dH <sub>2</sub> O	1.95 mL	1.7 mL	1.2 mL
Glycerol	-	800 $\mu$ L	800 $\mu$ L
10% w/v APS	62.5 $\mu$ L	12.5 $\mu$ L	12.5 $\mu$ L
TEMED	12.5 $\mu$ L	5 $\mu$ L	5 $\mu$ L

**Table 2.1: Composition of a 10 and 12% tris-tricine polyacrylamide gel.**

overnight in destain containing tissue to preferentially remove the dye. When sufficiently destained, the gels were visualised and photographed.

### 2.2.3 Native polyacrylamide gel electrophoresis

Non-denaturing polyacrylamide gels were used for gel retardation assays and stoichiometry studies at an 8% acrylamide concentration and for the purification of duplex DNA at a 16% acrylamide concentration. Two sizes of gels were used, the 12 × 10 cm plates that require 10 mL of gel solution were used for gel retardation assays and the 16 × 16 cm plates separated by 2 mm spacers that require 40 mL of gel solution were used for DNA purification. The plates, combs and spacers were thoroughly cleaned with dH<sub>2</sub>O then degreased with EtOH. The required volumes of gel solution were made with TBE (0.25 × TBE for the 10 mL gels, 1 × TBE for the 40 mL gels), 0.1% (w/v) APS and 0.2% (v/v) TEMED with the required percentage acrylamide from 40% AccuGel™ 19:1. The solution was poured and allowed to polymerise overnight at 4°C. The gel was then pre-electrophoresed in a TBE buffer of the same concentration as the gel for 1 h at 4°C, 100 V for the 10 mL gels and 200 V for the 40 mL gels. The sample loading buffers and gel running times are detailed under subsequent sections. The gels were visualised using EtBr for unlabelled DNA.

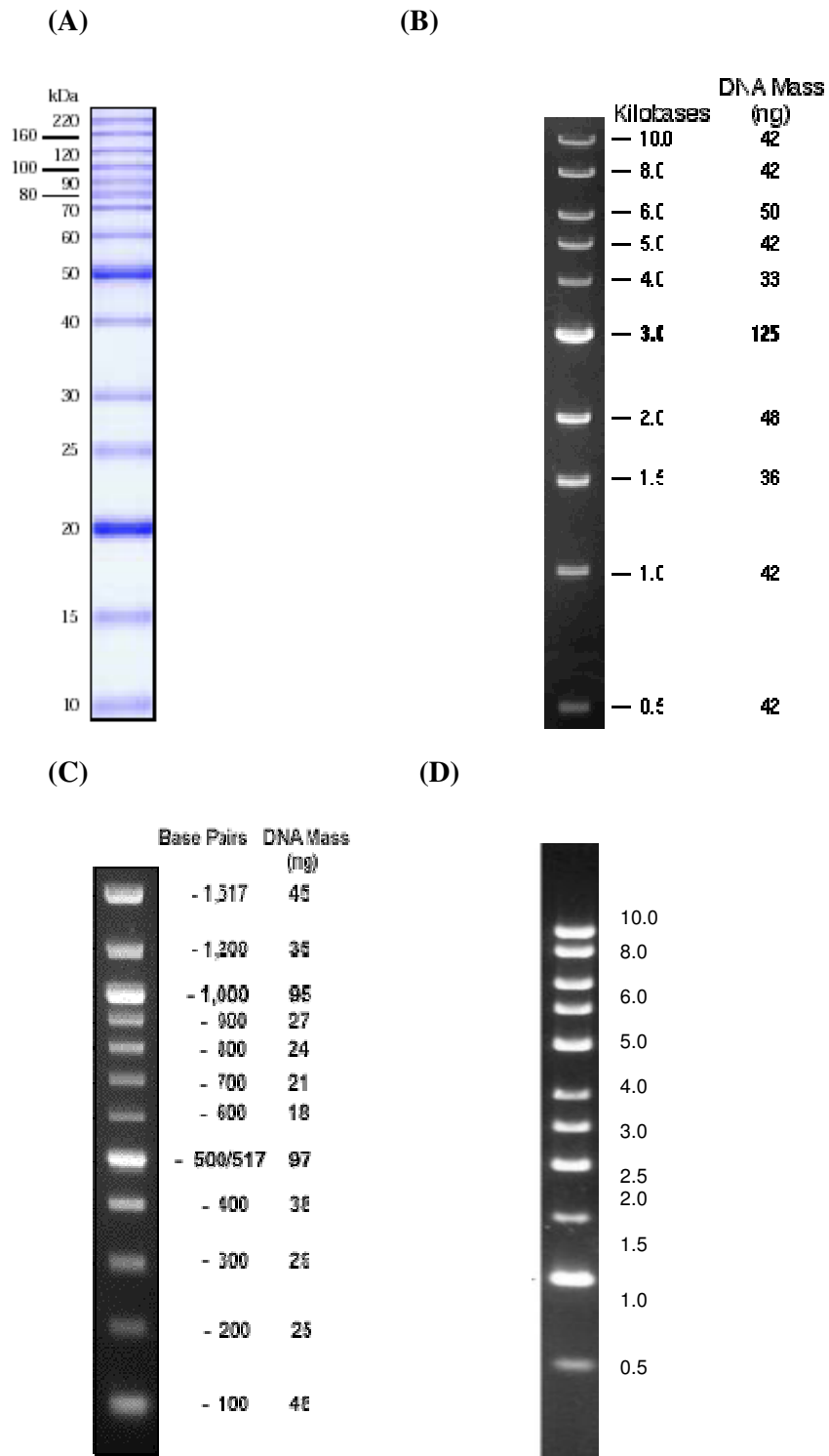
## **2.3 Sub-cloning**

### 2.3.1 Plasmid preparation

Small quantities of the plasmid were prepared using the QIAprep® Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

### 2.3.2. Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) was used for the amplification of the R gene from the parental clone containing the entire EcoR124I system in the pCP1005 vector. All PCR reactions were performed using the standard PCR master mix including Vent DNA polymerase (NEB) as it contains 3' to 5' proofreading, as opposed to Taq DNA polymerase. The extension time was



**Figure 2.1: Four gel markers used for protein and DNA analysis.** (A) is a Benchmark<sup>TM</sup> protein ladder (Invitrogen). (B) is a 1 kbp DNA ladder (NEB). (C) is a 100 bp DNA ladder (NEB). (D) is a 1 kbp DNA ladder (Amersham Biosciences).

based on 1 kb of PCR product produced per 60 seconds of incubation. The PCR mixtures were placed into a PTC-150 Minicycler™ PCR machine (MJ Research) with a hot bonnet attachment to minimise condensation and the following program was run: melting (5 min, 95°C), followed by 25 cycles of melting (30 s, 95°C), annealing (30 s, 55°C) and extension (3 min 15 s, 72°C). The samples were then run on an agarose gel (typically 2%), the appropriate band was excised after visualisation using a low energy UV transilluminator, and the DNA was purified using the QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

### 2.3.3 Restriction enzyme digests

The plasmid and PCR products were digested using restriction enzymes and the reactions were performed according to the manufacturer's instructions. The PCR product was then purified using the QIAquick® PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

### 2.3.4 Purification of DNA

Following PCR, the products were purified by using either the Qiaquick® PCR Purification kit or the bands of interest were removed using a clean scalpel blade and purified using a QIAEX II® Agarose Gel Extraction Kit (Qiagen).

### 2.3.5 TOPO® TA cloning reaction

Following PCR, the products were incubated with 0.2 µL of Taq DNA polymerase at 5 units/µL to add 3' A overhangs, for 30 mins at 72°C. 1 µL of this reaction was used in the TOPO® TA cloning reaction [1 µL fresh PCR reaction, 1 µL salt solution (1.2 M NaCl, 0.06M MgCl<sub>2</sub>), 1 µL TOPO® vector and 7 µL sterile water]. The reaction was left at 22°C for 30 minutes. 2 µL of the TOPO® TA cloning reaction was transformed into Mach1™- T1® *E.coli* competent cells (see section 2.4.2). Following plasmid-DNA preparation (see section 2.3.1), the plasmids were screened by restriction digestion (2.3.3).

### 2.3.6 Ligation reactions

Ligation of the gene into the vector DNA was carried out in a 14  $\mu\text{L}$  reaction mixture containing 0.5  $\mu\text{L}$  (200 U) T4 DNA ligase and 1  $\times$  T4 DNA ligase reaction buffer. The reaction mixture was incubated at 16°C overnight. The reaction was performed using a 5:1 insert to plasmid ratio. Following incubation, the product was used to transform competent *E. coli* JM109 cells.

## **2.4 Protein expression**

### 2.4.1 Competent cell production

Bacterial cells are required to be competent to enable effective uptake of plasmid vectors or plasmid vector constructs. For this purpose, strains of *E. coli* [JM109, DH5 $\alpha$  and BL21 (DE3) Gold] were plated onto fresh agar containing no antibiotic. A single colony was then used to inoculate 10 mL 2  $\times$  YT that was grown overnight at 37°C. The overnight culture was used to inoculate 500 mL 2  $\times$  YT (pre-warmed to 37°C) and this was incubated at 37°C whilst shaking at 225 rpm. When an OD<sub>600</sub> of approximately 0.6 was achieved, the cells were collected by centrifugation in a swing out rotor at 4000 g, 4°C for 20 min and the cells were resuspended in 166 mL FB1 and left on ice for 15 min. The centrifugation step was repeated, the cells were resuspended in 40 mL FB2 and kept on ice for a further 15 min. Aliquots of 1 mL were then transferred to microcentrifuge tubes on ice, snap frozen in liquid N<sub>2</sub>, and stored at -70°C until required.

### 2.4.2 Transformation of competent cells

A 1 mL aliquot of competent cells was thawed on ice and 100  $\mu\text{L}$  transferred to a cold microcentrifuge tube, to which either 1  $\mu\text{L}$  plasmid or 14  $\mu\text{L}$  ligation product was added. The tube was kept on ice for 10 min, heat shocked at 42°C for 45 s and returned to ice for a further 2 min. A 900  $\mu\text{L}$  volume of cold 2  $\times$  YT was added to the tube, which was then placed in a shaking incubator at 37°C for 1 h. Centrifuging for 10 seconds pelleted the cells and 800  $\mu\text{L}$  supernatant was removed. The pellet was then resuspended in the remaining supernatant and 100  $\mu\text{L}$  was removed and spread onto an agar plate containing 50  $\mu\text{g mL}^{-1}$  ampicillin.

The plate was then incubated overnight at 37°C. The plates were discarded as soon as successful overnight starter cultures had been grown.

#### 2.4.3 Starter cultures

In the case of R and M expression, a single colony of transformed bacterial cells (section 2.4.2) from a plate was added to a 5 mL volume of 2 × YT medium containing 100 µg mL<sup>-1</sup> ampicillin for both expression of R and M. For S3 and S11, a single colony was added to a 100 mL volume of 2 × YT medium containing 50 µg mL<sup>-1</sup> kanamycin. In the case of S3 and S11, the cultures were incubated overnight at 37°C at 200 rpm in a shaking incubator. For expression of M and R, the cultures were grown at 37°C at 200 rpm until the OD<sub>600</sub> light scattering reading reached 0.6, as it was found growing the starter cultures resulted in loss or no expression of the target gene.

#### 2.4.4 Preliminary expression tests and large-scale protein expression

Expression testing and large-scale expression of the native target protein was carried out in 2 L culture flasks containing 0.5 L 2 × YT or LB broth containing either 50 µg mL<sup>-1</sup> kanamycin (S11 and S3) or 100 µg mL<sup>-1</sup> ampicillin (M and R). The culture flasks were pre-warmed to 37°C and then inoculated with an overnight culture (100 mL for S11 and S3, and 5 mL for M and R). The flasks were incubated at 37°C in a shaking incubator (250 rpm) for approximately 2.5-3 h until the cells had reached mid-log phase, interpreted as an OD<sub>600</sub> light scattering reading of 0.6. Protein expression was then induced by the addition of 1 mM IPTG (in all cases) to the culture flasks and incubation continued for a further 3 h. The cells were harvested by centrifugation at 7000 rpm, 4°C for 30 min. The cell pellets were stored at -20°C.

### **2.5 Protein purification**

#### 2.5.1 S11 and S3

##### *2.5.1.1 Cell lysis*

Cell pellets were resuspended in 5 mL lysis buffer per 1 L of culture, at 4°C. The cells were then lysed using a Vibracell™ VCX 500 high intensity ultrasonic

processor (Jencons-PLS) with the CV 33, ½-inch probe attachment in a glass beaker set in ice. The amplitude typically was 40% for a 20 mL volume, and the temperature probe was inserted into the sample to ensure the temperature remained below 9°C. The cells were lysed using two cycles of 23 × 9.9 s sonic bursts interspersed with 9.9 s resting periods. Following lysis, the supernatant was separated from the cell debris by centrifugation at 39,000 g, 4°C for 30 min.

#### *2.5.1.2 Affinity Chromatography*

All further purification steps were carried out at 4°C. A 1 mL HiTrap chelating HP column (Amersham Pharmacia) was prepared by syringing through 10 mL water, followed by 2 mL 100 mM NiSO<sub>4</sub>·6H<sub>2</sub>O and 10 mL water. The column was connected to either an ÄKTA prime or ÄKTA purifier. The column was equilibrated with lysis buffer at 1 mL/min. 20 mL of clarified S3 following centrifugation was loaded at 1 mL/min. The column was washed through with 15 CV lysis buffer. A 15 CV gradient of 0-100 % elution buffer was run at 1 mL/min. S11 or S3 eluted at approximately 200 mM imidazole. 100 % elution buffer was then run at 1 mL/min for 15 CV. The yield at this stage is typically 16 mg/L bacterial cells

#### *2.5.1.3 Cleavage of Histidine-tag*

Following affinity chromatography, the pooled fractions were buffer exchanged using a 25 mL Vivaspin (MWCO 5 kDa for S11 or 10 kDa for S3) or dialysed overnight into IEX1 pH 8.0. The thrombin cleavage reaction was performed at room temperature for two hours using an enzyme concentration of 2 U/mg protein. Cleavage was found not to require CaCl<sub>2</sub>. The reaction was stopped with 1 mM PMSF.

#### *2.5.1.4 Affinity Chromatography*

A 1 mL HiTrap chelating HP column (Amersham Pharmacia) was stripped with and recharged with nickel ions. The procedure was then identical to section 2.5.1.2, except the flow through was collected containing native S11 or S3. No further purification was required.

## 2.5.2 The M subunit

### *2.5.2.1 Cell lysis*

Cell pellets were resuspended in 20 mL lysis buffer per 1 L of culture, at 4°C. The cells were then lysed using a Vibracell™ VCX 500 high intensity ultrasonic processor (Jencons-PLS) with the CV 33, ½-inch probe attachment in a glass beaker set in ice. The amplitude was typically 30% for a 50 mL volume, and the temperature probe was inserted into the sample to ensure the temperature remained below 9°C. The cells were lysed using two cycles of 15 × 9.9 s sonic bursts interspersed with 9.9 s resting periods. Following lysis, the supernatant was separated from the cell debris by centrifugation at 39,000 g, 4°C for 30 min.

### *2.5.2.2 Removal of DNA*

Following the removal of insoluble macromolecules and cell debris, DNA was removed. To the lysate, an equal volume of a buffer containing 20 mg/mL protamine sulphate salt from salmon (Sigma), 1 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT, was added such that the final concentration of NaCl was 500 mM and protamine sulphate was 10 mg/mL whilst slowly mixing at 4°C. The solution was mixed at 4°C for 30 minutes before precipitated nucleic acids were removed from the sample by centrifugation (12,000 g, 4°C, 20 min). and then resuspended in the appropriate volume of heparin buffer A, typically 5 mL per pellet. The sample was then dialysed overnight in IEX 1. Before column loading, the sample was centrifuged (27,000 g, 4°C, 30 min).

### *2.5.2.3 Desalting*

Four 5 mL HiTrap desalting columns were connected in series and equilibrated in IEX1 buffer. 1.5 mL of sample (following protamine sulphate precipitation) was injected. The flow-rate was 5 mL min<sup>-1</sup> and the fraction size was 1.8 mL. The appropriate fractions were analysed by 12.5 % SDS-PAGE, and the appropriate fractions were then pooled and dialysed overnight in IEX1 buffer.

### 2.5.3. The R subunit

#### *2.5.3.1 Cell lysis*

Cell pellets were resuspended in 20 mL lysis buffer per 1 L of culture, at 4°C. The cells were then lysed using a Vibracell™ VCX 500 high intensity ultrasonic processor (Jencons-PLS) with the CV 33, ½-inch probe attachment in a glass beaker set in ice. The amplitude was set to equal the lysis volume, typically 30% for a 50 mL volume, and the temperature probe was inserted into the sample to ensure the temperature remained below 9°C. The cells were lysed using two cycles of 15 × 9.9 s sonic bursts interspersed with 9.9 s resting periods. Following lysis, the supernatant was separated from the cell debris by centrifugation at 39,000 g, 4°C for 30 min.

#### *2.5.3.2 Removal of DNA*

Following the removal of insoluble macromolecules and cell debris, DNA was removed. To the lysate to a final concentration of 20 mg/mL protamine sulphate salt from salmon (sigma) in was then added in a buffer containing 1 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT, by adding an equal volume, such that the final concentration of NaCl was 500 mM whilst slowly mixing at 4°C. The solution was mixed at 4°C for 30 minutes before precipitated nucleic acids were removed from the sample by centrifugation (12,000 g, 4°C, 20 min). The sample was then dialysed overnight in IEX 2. Before column loading, the sample was centrifuged (27,000 g, 4°C, 30 min) on a HiTrap heparin column.

#### *2.5.3.3 Heparin Chromatography*

Heparin and all subsequent chromatographic steps were performed on the ÄKTA Purifier system (Amersham Pharmacia) using columns purchased from Amersham Pharmacia. Heparin FPLC was performed using a four 1 mL HiTrap™ heparin HP columns connected in series and consisted of equilibrating the column with 5 column volumes (CV) IEX3 buffer, injecting the sample (typically 10-12 mL), 12 CV wash/equilibration and a 15 CV linear gradient (0-100% IEX4 buffer B). The flow-rate was 1 mL min<sup>-1</sup> and the fraction size was 2 mL. The appropriate fractions were then pooled and dialysed overnight in IEX3 buffer.

#### 2.5.3.4 Mono Q

SP Sepharose FPLC was performed using a 0.9 mL Mono Q HR 5/5 chromatography column (Amersham Biosciences) and consisted of equilibrating the column with 5 CV IEX3, injecting the filtered dialysed sample, 10 CV equilibration with IEX3, a 30 CV linear gradient (0-30% IEX4 buffer B) and a 5 CV column clean (100% SP Sepharose buffer B). The flow-rate was 1 mL min<sup>-1</sup> and the fraction size was 1.8 mL. The appropriate fractions from both runs were then pooled and dialysed overnight in IEX3 buffer.

#### 2.5.4. Complexes of S3/M and S3/M/R

Complexes of S3/M and S3/M/R were formed following the individual expression and purification of the subunits of the EcoR124I<sub>NT</sub> system, as described in sections 3.5 and 3.6.

#### 2.5.5 Preparative size exclusion chromatography

Size exclusion chromatography of S11, S3, M or S3/M was performed using a Superdex 12 high resolution column and consisted of equilibrating the column with 1.5 CV IEX1 before running the program, a further 0.01 CV equilibration, injecting the filtered sample (typically 200 µL) and a 1.5 CV elution step. The flow-rate was 0.4 mL min<sup>-1</sup> and the fraction size was 1.8 mL. The appropriate fractions were then pooled.

#### 2.5.6 Sample concentration

Protein Samples could be concentrated using 2 mL and 20 mL Vivaspins (Sartorius group). However some loss of sample was evident, therefore two different types of concentrators by Millipore were used in later protein purifications and resulted in little loss of samples. For larger volumes, the Centricon Plus-70 (Millipore) was used and for smaller volumes up to 15 mL, Centriprep YM-30 centrifugal filter (Millipore) were used. Table 2.2, lists the type of concentrators used for each protein.

### 2.5.7 Protein storage

All proteins samples could be stored at 4°C for at least two weeks. In the case of S11, S3 and R, the proteins were able to be stored at -20°C in 50% glycerol for longer-term storage. Complexes of S3/M and S3/M/R were prepared fresh prior to experiments as described in sections 3.5 and 3.6

## **2.6 UV spectroscopy**

Calculation of DNA and protein concentrations was performed from UV absorption spectra obtained using 1 mL quartz cuvettes in a Perkin Elmer Instruments Lambda 25 UV-Vis Spectrometer scanning from 350 nm to 220 nm.

### 2.6.1 DNA concentration

A reasonable estimation of the DNA concentration is given by the approximation that for double-stranded DNA, an OD of 1.0 at 260 nm is equivalent to a concentration of 50  $\mu\text{g mL}^{-1}$ . However, this method assumes that there is no sequence-dependent change in DNA hyperchromicity.

### 2.6.2 Protein concentration

Protein concentration was estimated using Beer-Lambert's Law,  $\text{OD} = \epsilon cl$ , where  $\epsilon$  = the molar extinction coefficient for the protein calculated using the ProtParam tool (Gill and von Hippel, 1989),  $c$  = the molarity and  $l$  = the path length of the cuvette in centimetres (usually 1 cm).

## **2.7 DNA duplex formation and purification**

### 2.7.1 Duplex formation

Approximately equimolar amounts of the synthetic complementary oligonucleotides were mixed together and heated to a temperature of 90°C for 10 min in annealing buffer. The solution was cooled to approximately 20°C and orange G loading dye was added. The samples were then loaded onto a 16% native gel (section 2.2.3) and run for 3 hr to confirm the duplex had formed.

(A)

	<b>Vivaspin (Sartorius)</b>
<b>Sample</b>	<b>MWCO (kDa)</b>
<b>S11</b>	5
<b>S3</b>	10
<b>M</b>	30
<b>R</b>	30
<b>S3/M</b>	30
<b>S3/M/R</b>	30

(B)

	<b>Millipore</b>	
<b>Sample</b>	<b>Centricon Plus-70 MWCO (kDa)</b>	<b>Centriprep YM-30 centrifugal filter MWCO (kDa)</b>
<b>S11</b>	3	3
<b>S3</b>	10	10
<b>M</b>	10	10
<b>R</b>	30	30
<b>S3/M</b>	30	30
<b>S3/M/R</b>	30	30

**Table 2.2: Concentrators used for the concentration of samples of the EcoR124I<sub>NT</sub> system.** (A) 2 mL and 20 mL Vivaspins (Sartorius) concentrators used (B) 75 mL Centricon Plus-70 (Millipore) and 15 mL Centriprep YM-30 centrifugal filter (Millipore) concentrators used.

### 2.7.2 Ethanol precipitation

Ethanol precipitation of DNA was carried out by the addition of 0.1 volumes of 3 M sodium acetate pH 5.2 and 3 volumes of ice-cold absolute EtOH to the solution containing the target DNA following annealing. The solution was placed at -20°C overnight before the samples were centrifuged at 27,000 g, 4°C for 30 min. The EtOH was aspirated from the tube and replaced with 1 mL 70% EtOH to remove contaminating salt from the DNA. The sample was centrifuged at 16,000 g, 4°C for 30 min and the EtOH was again aspirated. The samples were then allowed to dry horizontally at 40°C in a heating block, resuspended in annealing buffer and the concentration estimated (section 2.6.1).

## **2.8 Analytical ultracentrifugation (AUC)**

A Beckman Optima XL-A analytical ultracentrifuge (Beckman-Coulter, Palo Alto, CA, USA) was used for sedimentation equilibrium experiments. All measurements were recorded at 10°C for sedimentation velocity and 4°C or 20°C for sedimentation equilibrium. The sedimentation equilibrium data was analysed using XL-A/XL-I Data Analysis Software Version 6.03 (Beckman Coulter) within Origin 6.0 (Microcal Software, Inc.). The sedimentation velocity data was analysed using Sedfit (Schuck *et al.*, 2000).  $\bar{v}$  values were corrected for temperature (table 2.3). Solvent density and viscosity values were calculated using Sednterp (Laue *et al.*, 1992) and are shown in table 2.4.

### 2.8.1 Protein sedimentation equilibrium

The experiment was performed in six-channel cells of 12 mm optical path length, using 90  $\mu$ L of sample at a range of protein concentrations. 100  $\mu$ L of buffer was loaded into the corresponding control channel. The cells were loaded into an AN50-Ti analytical rotor, which had been left overnight at 4°C and transferred to the centrifuge, where it was left to equilibrate to the appropriate temperature. The rotor was accelerated to 3,000, 6,500, 8,500, 18,000 and 40,000 rpm and scans of absorbance versus radial displacement were taken at a resolution of 0.001 cm at 0, 15, 18 and 21 hr. The wavelengths which were scanned at are described in section 5.3.

### 2.8.2 Protein sedimentation velocity

400  $\mu\text{L}$  of sample and 425  $\mu\text{L}$  of buffer were loaded into the corresponding sectors of a double sector cell of 12 mm optical path length. The cells were loaded into an AN50-Ti analytical rotor, which had been left overnight at 4°C and transferred to the centrifuge, where it was left to equilibrate to 10°C. The rotor was accelerated to 30,000 rpm and readings of absorbance versus radial distance were taken every 12 minutes at 280 nm.

### **2.9 Dynamic light scattering**

Dynamic light scattering (DLS) was performed on a series of S11, S3, M, R, S3/M and S3/M/R concentrations at 4°C using a Protein Solutions DynaPro MSTC800 light scattering instrument. From the resulting hydrodynamic radius,  $R_h$ , an estimate of the molecular weight,  $M_r$ , of the protein was estimated using the empirical relationship for typical globular proteins:

$$M_r = (1.68 \times R_h)^{2.34}$$

### **2.10 Small angle neutron scattering**

SANS experiments were carried out at ILL, Grenoble, France with the assistance of Dr Phil Callow. S3 was deuterated by first adapting BL21 (DE3) cells expressing the S3 subunit in pET-21a in minimal media. Good expression was achieved (70 mg from 1L bacterial cells). The purification was essentially the same as for the protonated S3 subunit (see chapter 3).

The methyltransferase and endonuclease were formed in a number of protonated and deuterated states; i.e. protonated S3 and M or deuterated S3 and protonated M for the methyltransferase and for the endonuclease, protonated S3, M and R subunits or deuterated S3 with protonated M and R. In order to have fully hydrogenated, deuterated or perdeuterated subunits and complexes in two different  $\text{D}_2\text{O} / \text{H}_2\text{O}$  percentages (40 % and 100 %  $\text{D}_2\text{O}$ ), samples that had been dialysed into buffer (IEX) containing either 0 % or 100 %  $\text{D}_2\text{O}$  were mixed and

then left for a period of about 12 hours (table 2.5). Lyophilised DNA duplex was resuspended in an appropriate amount of 100 % D<sub>2</sub>O dialysis buffer and treated in the same way.

Complexes of S3/M/R were formed by the addition of either fully protonated S3/M or perdeuterated dS3/M. For the complexes in the presence of DNA, a concentrated 21.5 mM stock (to minimise the dilution of the complexes) of a 30 mer DNA duplex (S3-30) containing the symmetrical recognition sequence (GAAN<sub>7</sub>TTC), was added and left to incubate at 4 °C for at least 30 minutes prior to the experiment being carried out.

Firstly the beam centre was found so that all spectra could be aligned. An automated sample changer was loaded with either 1 mm or 2 mm pathlength quartz cuvettes holding either 400 µL or 200 µL of sample respectively. Measurements were carried out at 4 °C, using a thermostated water bath. Transmissions were measured for 10 minutes at distances of 2 and 8 m for an empty cell, a standard containing water and the background. Scattering data was collected from a 96 x 96 cm detector with a pixel size of 7.5 x 7.5 mm. Measurements were carried out for all experiments at a wavelength of 6 Å. For cells containing sample, data was collected for a period of 30 minutes for each experiment. Measurements were carried out at 8 m for the smaller angle measurements and at 2 m for the larger angle measurements.

## **2.11 Crystallisation trials**

The hanging drop and sitting drop vapour diffusion studies followed the methods of Davies and Segal (1971). The protein was buffer-exchanged into the crystal trials buffer. Hanging drop trays were set up at room temperature (20-25°C) with 500 µL precipitant solution in the well and two drops of 2 µL each of the precipitant solution on a glass cover slip. Two 2 µL drops, one containing protein, the other only consisting of the crystal trials buffer were then diluted into the drops of precipitant solution. The cover slips were inverted over the well containing the appropriate precipitant solution and sealed in place with vacuum

Sample	$\bar{V}$ values (mL/g) at T =			
	25 °C	20 °C	10 °C	4 °C
S11	0.7445	0.7423	0.7381	0.7356
S3	0.7415	0.7394	0.7351	0.7326
S3/M	0.7398	0.7377	0.7334	0.7309
S3/M/R1	0.7370	0.7349	0.7307	0.7277
S3/M/R2	0.7363	0.7341	0.7299	0.7273
S3/M plus DNA (1:1 molar ratio)	0.7183	0.7162	0.7119	0.7093
R	0.7344	0.7323	0.7280	0.7254

**Table 2.3:  $\bar{V}$  values corrected for temperature.** Values were calculated both using Sednterp, by entering the theoretical  $M_r$  and the calculated solvent density and viscosity for the appropriate buffer (table 2.4) and equation. The initial  $\bar{V}$  at 25 °C for S3M plus DNA was calculated using equation 14, before being corrected for temperature using equation 12, chapter 5.

Buffer	Solvent density, $\rho$ (g/mL), at T =				Solvent viscosity, $\eta$ (poise), at T =			
	25 °C	20 °C	10 °C	4 °C	25 °C	20 °C	10 °C	4 °C
10 mM sodium phosphate pH 8.0	1.00263	1.00379	1.00530	1.00557	0.90199	1.0150	1.3241	1.5872
IEX1 pH 8.0	1.00167	1.00283	1.00433	1.00461	0.90184	1.0148	1.3239	1.5870

**Table 2.4: Solvent density and viscosity values for each of the buffers used in sedimentation and sedimentation velocity experiments.** The buffer density and viscosity values were also corrected for temperature using Sednterp, assuming water is the greatest component.

Dialysis Buffer	Volume ( $\mu\text{L}$ ) of each sample / buffer used to obtain final $\text{D}_2\text{O}$ %		
	100 %	40 %	0 %
100 % $\text{D}_2\text{O}$	400	160	0
100 % $\text{H}_2\text{O}$	0	240	400

**Table 2.5: Volumes of each sample / buffer that had been dialysed into a buffer containing either 0 % or 100 %  $\text{D}_2\text{O}$  used to form the various  $\text{H}_2\text{O}/\text{D}_2\text{O}$  contrasts.**

grease. The trays were stored at 16°C and the results were visualised using a polarising light microscope with a mounted camera.

## **2.12 Methylation assay**

### 2.12.1 Substrate preparation

The substrate for the methylation assay pUC119/EcoR124I<sub>NT</sub> was formed from pUC119 EcoRI-, as described in section 4.2.2. For the large-scale plasmid preparation a HiSpeed™ Plasmid Maxi Kit (Qiagen) was used and the purified plasmid DNA was linearised with XmnI. The DNA concentration was measured by its UV absorbance spectrum at 260 nm.

### 2.12.2 Assay

The methylation assay was set up by mixing the methylation buffer and protein before the addition of the linearised pUC119/EcoR124I<sub>NT</sub> to start the reaction. Samples were incubated at 37 °C. During time course experiments, 15 µL aliquots were removed and heat inactivated at 65 °C for 20 minutes. After cooling on ice for 10 minutes each 15 µL was challenged with 4 units of EcoRI restriction endonuclease and left to incubate for a further 60 minutes at 37 °C. The products of the reaction were run on a 0.8 % agarose gel.

The OCR inhibition assay was carried out in the same way, except OCR was added at the appropriate molar ratio following addition of S3/M to the DNA.

### 2.12.3 Gel analysis

The gel was digitally photographed using either the BioRad gel documentation system or the FujiFilm FLA-5000 phosphoimager and scanned using the Image Reader FLA-5000 version 3 programme before quantification using the Image Gauge version 4.2.1 programme (both part of the ScienceLab 2001 software package). The fluorescence settings used were the red filter (position 4) using the 532 nm green laser at 800V, with the one laser / one image setting.

## **2.13 Restriction endonuclease assay**

### 2.13.1 Substrate preparation

The substrates for the restriction endonuclease assays are described in table 4.2. For the large-scale plasmid preparation a HiSpeed™ Plasmid Maxi Kit (Qiagen) was used to purify the starting plasmid-DNA substrates, before linearisation if required with the appropriate Type II restriction endonuclease. The DNA concentration was measured by its UV absorbance spectrum at 260 nm.

### 2.13.2 Assay

The restriction endonuclease assays were set up by mixing the appropriate buffer, firstly with S3/M for 10 minutes at 37°C, before the addition of a 2:1 molar ratio of R:S3/M (typically 200 nM to 10 nM) to form the S3/M/R complex, which was then left for a further 10 minutes at 37°C, before the addition of the DNA substrate, in all cases at a final concentration of 10 nM. The reactions were started with the addition of 2 mM ATP (or not for those reactions without ATP). Samples were incubated at 37 °C. During time course experiments, which were usually carried out 60 minutes, 15 µL aliquots were removed and heat inactivated at 65 °C for 20 minutes. The products of the reaction were run on a 0.8 % agarose gel.

### 2.13.3 Gel analysis

The gel was digitally photographed using either the BioRad gel documentation system ensuring there were no saturated pixels. A graphical representation of each lane was produced by densitometry and exported in Excel.

## **2.14 DNA Sequencing**

DNA sequencing was performed by Cytomyx or Lark Technologies using custom designed primers or their own stock primers.