D. METHODS

1. Small and midi scale isolation of plasmid DNA from *E. coli* (Sambrook *et al.*, 1989; Birnboim and Doly, 1979)

For small scale preparation of plasmid DNA 3 ml of LB medium containing the proper antibiotic supplement are inoculated with a single bacterial colony and incubated overnight in a shaker at 37°C. On the next day 1.5 ml of the bacterial culture are centrifuged, the supernatant is discarded and the pellet is resuspended in 100 µl of ice cold solution I (25 mM Tris-HCl pH 7.8; 50 mM Glucose; 1 mM EDTA). After 5 min incubation at room temperature, 200 µl of solution II (0.2 N NaOH; 1% SDS) are added, the sample is mixed carefully and incubated for 5 min on ice. Cellular proteins and chromosomal DNA are then precipitated by addition of 150 µl of solution III (3 M sodium acetate pH 4.8) and a further 5 min incubation on ice. Cell debris is removed by centrifugation (5 min; RT; 12000 rpm) and the supernatant is first extracted with a mixture of phenol/ chloroform (1:1) and then precipitated by addition of 1 ml ethanol. After 2 min incubation at room temperature the precipitated plasmid DNA is centrifuged (5 min; RT; 12000 rpm), dried in the speed vac for 5-10 min and resuspended in 20 µl TE (10 mM Tris-HCl pH 8.0; 1 mM sodium EDTA) containing 20 µg/ml RNase A.

For midi scale plasmid preparations 25 ml (with high copy plasmids) or 100 ml (with low or medium copy plasmids) LB medium supplemented with the proper antibiotic are inoculated with a single bacterial colony and incubated over night at 37 °C in a shaker. Bacteria are then pelleted by centrifugation (JA20; 8000 rpm; 4 °C; 10 min) and plasmid DNA is isolated using the midi scale plasmid DNA extraction kit (Qiagen Corp.) according to the manufacturer's instructions.

2. Small and midi scale preparation of chromosomal DNA from *Helicobacter pylori* (Gross and Rappuoli, 1989)

For small scale isolation of *H. pylori* chromosomal DNA cells from one plate are resuspended in 1.5 ml STE (10 mM Tris-HCl pH 7.8; 100 mM NaCl; 1 mM EDTA), collected by centrifugation and resuspended again in 350 µl STE. Two µl of lysozyme solution (100 mg/ml in STE) and 4 µl of RNAse A solution (10 mg/ml in H_2O) are then added and the sample is incubated for 10-20 min at 37 °C. Thereupon 17.5 µl of 10% SDS are added and incubation is continued for another 15 min at 65 °C. After that the sample is mixed with 1 µl of proteinase K solution (100 mg/ml in STE) and

incubated at 50 °C for at least 2 hours. The chromosomal DNA is then extracted twice with an equal amount of phenol/chloroform (1:1), and precipitated by the addition of 2.5 volumes ethanol. After 20 min at -20 °C, the precipitated DNA is collected by centrifugation (10 min; 4 °C; 12000 rpm) washed once with 75% ethanol, air-dried and solubilised in TE buffer (10 mM Tris-HCl pH 8.0; 1 mM sodium EDTA).

For midi scale preparation of *H. pylori* chromosomal DNA cells from 6-10 plates are resuspended in 10 ml of solution I (50 mM Tris-HCl pH 8.0; 50 mM EDTA) and incubated for at least 30 min at -20 °C. After the addition of 1 ml lysozyme (10 mg/ml) cells are rethawed slowly and incubated for a further 45 min on ice. Six ml of solution II (50 mM Tris-HCl pH 8.0; 400 mM EDTA; 0.5% SDS) as well as 1 ml of proteinase K (20 mg/ml in H_2O) are then added and incubation is continued for 1 hour at 50 °C. Chromosomal DNA is extracted three times with an equal volume of phenol/chloroform (1:1) and once with an equal volume of chloroform and then precipitated by the addition of 1/10 volume 5 M NaCl and 2 volumes ethanol. After incubation on ice for 15 min the DNA is sedimented by centrifugation (5000 rpm; 4 °C; 15 min; JA 20), dried, and resuspended in solution III (50 mM Tris-HCl pH 8.0; 1 mM EDTA). RNAse A (10 mg/ml in H_2O) is added to a final concentration of 200 µg/ml and the sample is incubated over night at 4 °C for RNA digestion. On the next day the chromosomal DNA is re-extracted two to three times with phenol/chloroform (1:1) and once with chloroform and re-precipitated with 1/10 volume 5 M NaCl and 2 volumes ethanol. The precipitated DNA is then once again sedimented by centrifugation (8000 rpm; 15 min; 4 °C; JA 20), air-dried and resuspended in 1 ml of TE buffer (10 mM Tris-HCl pH 8.0; 1 mM sodium EDTA).

3. Cleavage of DNA with restriction endonucleases (Sambrook et *al.*, 1989)

The desired amount of chromosomal or plasmid DNA is diluted to a concentration of approximately 50-100 ng/µl in 1x restriction buffer (supplied 10x by manufacturers) and incubated for at least one hour at 37 °C with the appropriate amount (1 Unit per µg DNA) of restriction endonuclease. The precise reaction conditions and the composition of the different 10x reaction buffers are illustrated in the manufacturer's catalogues.

In the case of analytical cleavage the reaction can be stopped by addition of 0.5 vol stop buffer (0.01% bromophenol blue; 50% sucrose; 0.25 mM EDTA) and the DNA fragments can be loaded directly on an agarose gel. For further treatment of the cleaved DNA with modifying enzymes (e.g. alkaline phosphatase, DNA-ligase) the reaction is stopped by heat inactivation of the restriction enzyme for 10 min at 68 °C. Non-heat sensitive enzymes can alternatively be removed by extraction with a mixture of phenol/chloroform (1:1). After heat inactivation or phenol extraction, respectively, the cleaved DNA is precipitated with 1/10 vol 5 M NaCl and 2 vol ethanol and resuspended in an appropriate volume of H_2O or TE.

4. Filling in of DNA ends with klenow enzyme (Sambrook *et al*., 1989)

In order to fill in DNA 5'overhangs after cleavage with restriction endonucleases, the digestion reaction is supplemented with 150 µM dNTP mix and 1 µl klenow enzyme (5 U/µl) and incubated for 30 min at room temperature. After that the enzymes are either heat inactivated by a 10 min incubation at 75 °C or removed by extraction with phenol/chloroform (1:1).

5. 5'-dephosphorylation of DNA ends with alkaline phosphatase (Sambrook *et al*., 1989)

For dephosphorylation of DNA 5' ends 1-10 µg of the DNA fragment of interest are resuspended in 50 µl 1x CIP buffer (50 mM Tris-HCl pH 9.0; 1 mM $MgCl_2$; 0.1 mM $ZnCl_2$; 1 mM spermidine), mixed with 1 µl calf intestinal phosphatase (2-3 U/µl) and incubated for 30 min at 37 °C. With blunt ends after this period another 1 µl of alkaline phosphatase is added and incubation is continued for further 30 min at 56 °C. The enzyme is then extracted once with 1 volume of phenol/chloroform (1:1) and once with 1 volume of chloroform, and the dephosphorylated DNA is pecipitated by the addition of 1/10 volume 5 M NaCl and 2.5 volumes ethanol. After centrifugation and drying, the DNA is resuspended in 10-20 µl of TE.

If the dephosphorylation is to be carried out directly after the treatment with restriction endonucleases, the digestion reaction is simply mixed with 1/10 volume 1 M glycine pH 9.5 and 1 µl calf intestinal phosphatase and incubated at 37 °C and/or 56 °C. Phenol/chloroform extraction and ethanol precipitation is then performed as described above.

6. Polymerase chain reaction

For amplification of DNA sequences with the polymerase chain reaction (PCR) 10-100 ng of plasmid or chromosomal DNA are added to a reaction mixture containing 1x PCR buffer (Boehringer Mannheim), 200 µM dNTPs and 4 ng/µl of each specific oligonucleotide. One µl Taq polymerase (Boehringer Mannheim) is then added and PCR is performed by denaturing at 94 °C for 1 min, annealing at appropriate temperatures calculated on the basis of the melting temperature $T_M = ½(\{A+T\}x\ 2 + \{G+C\}x\ 4)$ of the respective oligonucleotides for 1 min and extending at 72 °C for 1 min. A total of 30 cycles is usually performed.

7. Deprotection of oligonucleotides

Oligonucleotides used for PCR, sequencing, and primer extension reactions are synthesised in an automatic DNA synthesiser according to the phosphoamidite method and have therefore to be deprotected before use. For that purpose they are solubilised in 2 ml ammonium hydroxide and incubated for 12-16 hours at 55 °C. After that, oligonucleotides are precipitated with 1/10 volume 3 M sodium acetate pH 5.2 and 2.5 volumes ethanol, collected by centrifugation (12000 rpm; 10 min; 4 °C), washed once with 75% ethanol, dried in the speedvac and resuspended in 100 µl of H_2O or TE.

8. Analysis of DNA fragments by agarose gel electrophoresis (Sambrook *et al.*, 1989)

For separation of DNA fragments larger than 100 bp the horizontal agarose gel electrophoresis is routinely used. Agarose concentrations vary between 0.5% and 2.0%, according to the size of the fragments of interest.

The desired amount of agarose is melted by boiling in 100 ml of 1x TBE (90 mM Tris-HCl pH 8.3; 90 mM boric acid; 2.5 mM EDTA), cooled down to approx. 50 °C, mixed with 3 µl ethidiumbromide (10 mg/ml), and poured into a tape-sealed electrophoresis support containing two combs. Once the gel is solidified the tape is removed and the support containing the gel is introduced into the electrophoresis chamber where it is covered with 1x TBE. The combs can then be removed and the resulting wells can be loaded with the restriction reactions. A power supply is connected to the electrophoresis chamber (anode at the plug opposite the wells) and electrophoresis is carried out at 5 V/cm for 1-2 hours. The separated DNA fragments can then be visualised under UV light.

9. Southern Blot (Southern, 1975)

After electrophoresis the DNA fragments are first depurinated by soaking the agarose gel for 30 min in two changes of 0.25 M NaOH with constant, gentle agitation. The gel is then rinsed extensively with H_2O, and denaturation of the DNA is carried out by incubating in an excess volume of denaturation solution (1.5 M NaCl; 0.5 M NaOH) for 20 min. After rinsing again with H_2O the gel is neutralised by a 20 min incubation in neutralisation solution (1 M Tris-HCl pH 7.4; 1.5 M NaCl), re-rinsed and soaked for 20 min in 20x SSC (3 M NaCl; 300 mM sodium citrate pH 7.0). It is then transferred to a clean glass plate and covered in a bubble-free manner with a

nitrocellulose filter of the same size that had been soaked in 20x SSC. Three equally soaked pieces of 3MM Whatman paper are placed on top of the nitrocellulose filter, followed by another 20 to 30 dry pieces of Whatman paper and 50 pieces of dry paper towel of the same size. A glass plate is then placed on top of the stack and weighed down with a 500 g weight. After the DNA transfer has occurred (8 to 24 hours after set up of the capillary transfer) the nitrocellulose filter is removed and the bound DNA fragments are crosslinked by baking the filter at 80 °C for two hours. Hybridisation of the DNA fragments with specific probes, as well as detection of the signal, is then carried out by using the ECL^{TM} direct nucleic acid labeling and detection system (Amersham) following the manufacturer's instructions.

10. Isolation of DNA fragments from agarose gels

Purification of DNA fragments or PCR amplification products from agarose gels is carried out by using the QiaEX DNA purification kit (Qiagen Inc.) following the manufacturer's instructions.

11. Cloning of DNA fragments (Sambrook *et al*., 1989)

The ligation of a DNA fragment with a plasmid vector is possible only if both DNA molecules possess compatible ends. At first the vector is therefore digested with such restriction endonucleases that produce the same 5' and 3' ends, that are present in the fragment. 0.1-0.3 µg of the linearised vector DNA are then mixed with the three fold molar amount of DNA fragment and 2 U T4 DNA ligase in 10 µl of 1x T4 ligase buffer. After an incubation of 4 to 16 hours at room temperature the sample can be used directly for transformation of appropriate *E. coli* competent cells.

For ligation of blunt ends, equal molar amounts of linearized vector and DNA fragment are usually used and PEG 6000 is added to a final concentration of 15% (w/v) to the ligation reaction.

12. Preparation of competent cells of *E. coli* strains DH5α and BL21 (Cosloy and Oishi, 1973; Dagert and Ehrlich, 1979)

One hundred ml of LB medium are inoculated with 1 ml of an over night culture of the desired *E. coli* strain and incubated in a shaker at 37 °C. When the bacterial culture has reached an OD at 590 nm of 0.4-0.6 the cells are harvested by centrifugation (JA20; 6000 rpm; 10 min; 4 °C), washed once with 60 ml ice cold 50 mM $CaCl_2$, and resuspended in 100 ml of the same solution. After 30

min incubation on ice the cells are recentrifuged (JA20; 6000 rpm; 10 min; 4 °C) and resuspended in 20 ml of 50 mM $CaCl_2$ / 20% glycerol. Cells are now competent and can either be transformed immediately or stored in form of 200 µl aliquots at –80 °C until further usage.

13. Transformation of *E. coli* DH5α and BL21 (Cosloy and Oishi, 1973; Dagert and Ehrlich, 1979)

Two hundred microliter competent cells of the *E. coli* strains DH5α or BL21 are mixed with 0.1-0.3 µg plasmid DNA or the respective amount of a ligation reaction and incubated for 30 min on ice. The mixture is then heat shocked for 1 min at 42 °C and after that reincubated on ice for another 30 min. Thereupon 1 ml LB medium is added and samples are incubated at 37 °C for approximately 1 hour for expression of antibiotic resistance genes. One hundred microliter of the suspension are then plated on a selective agar plate and the remaining cells are harvested by a short (10 sec) centrifugation. After that 1 ml of the resulting supernatant is discarded and the cells are resuspended in the remaining volume of 100 µl. This suspension is finally plated on a selective agar plate as well. Both plates are then incubated over night at 37 °C for growth of bacterial colonies.

14. Transformation of *Helicobacter pylori* strains

Natural transformation of *H. pylori* is carried out by adding 1-5 µg of plasmid DNA to a spot of fresh bacteria incubated for approximately 5 hours at 37 °C. After an over night incubation at 37 °C, the bacteria are collected, streaked on selective agar plates and incubated for 3-4 days in a jar. Single colonies are then selected for further analysis.

15. Long term storage of bacterial strains

For conservation of an *E. coli* strain over a period of several years 1 ml of an over night culture of the strain of interest is mixed with 1 ml of LB medium / glycerol (1:1), and stored at -80 °C. *H. pylori* strains are conserved similarly in modified Brucella broth containing 25% glycerol.

16. ß-galactosidase assay (Miller, 1972)

E. coli or *H. pylori* cells grown in liquid cultures to an OD at 590 nm of 0.7-1.0 are harvested and resuspended in an equal amount of PBS (137 mM NaCl; 2.6 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4 pH 7.4). The bacterial suspension (0.1 - 0.3 ml) is then added to 0.9 ml Z-buffer (60 mM Na_2HPO_4; 40 mM NaH_2PO_4; 10 mM KCl; 1 mM $MgSO_4$; 50 mM β-mercaptoethanol; pH 7.0) and cells are lysed by addition of one drop toluene and subsequent incubation at 37 °C for 30 min with continuous shaking. For detection of β-galactosidase activity, 0.2 ml of ONPG (0.4 mg/ml) are added and incubation is continued for 45 min at 37 °C. The reaction is then stopped by addition of 0.5 ml 1 M Na_2CO_3 and optical densities at 420 nm and 550 nm are determined. Activities are expressed in Miller Units according to the following formula: Units = $(A_{420} - 1.75 \times A_{550})/(t \times v \times A_{590}) \times 1000$ with

A_{420}	absorption at 420 nm
A_{550}	absorption at 550 nm
t	reaction time in minutes
v	culture volume in ml used for the assay
A_{590}	absorption of the culture at 590 nm

17. Purification of recombinant proteins

For purification of recombinant $[His]_6$ tagged proteins overnight cultures of the *E. coli* strains harbouring the respective expression plasmids are diluted 1:100 in 100 ml LB medium, grown to an OD at 590 nm of 0.7 and then induced by addition of IPTG to a final concentration of 1 mM. After growth for 4 more hours cells are harvested (JA20; 8000 rpm; 10 min) and resuspended in 5 ml of buffer A (50 mM NaH_2PO_4 pH 8.0; 300 mM NaCl; 20 mM imidazole). Samples are then incubated for 30 min with 1 mg/ml lysozyme on ice, sonicated, and centrifuged to remove unsoluble material (JA20; 12000 rpm; 20 min). The supernatants are mixed with 1 ml Ni^{2+}-NTA agarose (Qiagen), that had previously been equilibrated with buffer A, and incubated for 1-2 hours at 4 °C under continuous shaking to allow binding of the $[His]_6$ tagged proteins to the Ni^{2+}-NTA agarose. One ml polypropylene columns (Qiagen) are then packed stepwise with the samples and washed three times with 5 ml buffer A to remove non-specifically binding proteins. Finally the hexahistidine-tagged proteins are eluted with 2x 0.5 ml buffer B (50 mM NaH_2PO_4 pH 8.0; 300 mM NaCl; 250 mM imidazole) and dialysed against two 500 ml changes of buffer C (50 mM NaH_2PO_4 pH 8.0; 300 mM NaCl; 50% glycerol). Purified proteins are stored at -20 °C.

18. Purification of the wild type and the C-terminal truncated form of the *E. coli* RNA polymerase α subunit (Tang *et al*., 1995, 1996)

The wild type and the C-terminal truncated α subunit of the *E. coli* RNA polymerase are purified from *E. coli* strain BL21 harboring plasmids pHTT7f1-NHα and pHTT7f1-NHα(1-235) (Table 3), respectively. Over night cultures of both strains are diluted 1:100 in 25 ml LB medium, grown to an OD at 590 nm of 0.7 and then induced by the addition of IPTG to a final concentration of 1 mM. After growth for a further 3 hours at 37 °C, the culture is harvested by centrifugation (8000 rpm; 10 min; 4 °C; JA20), and the cell pellet is resuspended in 2 ml of buffer A (20 mM Tris-HCl pH 7.9; 500 mM NaCl; 5 mM imidazole). Cells are then lysed by sonication and the lysate is cleared by centrifugation (12000 rpm; 15 min; 4 °C; JA20). Hexahistidine-tagged α and α(1-235), respectively, are precipitated by addition of $(NH_4)_2SO_4$ to 60%, collected by centrifugation and redissolved in 2 ml of buffer B (6 M guanidine hydrochloride; 20 mM Tris-HCl pH 7.9; 500 mM NaCl; 5 mM imidazole). The sample is then mixed with 1 ml of Ni^{2+}-NTA agarose (Qiagen) in buffer B and incubated for 1-2 hours at 4 °C under continuous shaking to allow binding of the histidine-tagged protein. One ml polypropylene columns (Qiagen) are then packed with the samples and washed twice with 5 ml buffer B and twice with 3 ml buffer B plus 30 mM imidazole to remove non-specifically binding proteins. Finally, the His-tagged α proteins are eluted with 2x 0.5 ml buffer B plus 300 mM imidazole, dialysed 24 hours against 1 l buffer C (50 mM Tris-HCl pH 7.9; 0.5 mM EDTA; 5% glycerol) concentrated with Centricon-10 (Amicon) to 100 µl, mixed with 100 µl glycerol and stored at -20 °C.

19. Analysis of proteins by SDS polyacrylamide gel electrophoresis (Laemmli, 1970)

SDS polyacrylamide gels used for separation of proteins according to their molecular weight consist of a 5% stacking gel and a 7 to 15% running gel. For preparation of the running gel the following reagents are mixed in a beaker (in ml):

	7%	**10%**	**12.5%**	**15%**
acrylamide : N,N'-bisacrylamide (30% : 0.8%)	5.83	8.4	10.4	12.5
2.55 M Tris-HCl pH 8.3	3.7	3.7	3.7	3.7
H_2O	15.075	12.505	10.505	8.405
10% SDS (w/v in H_2O)	0.25	0.25	0.25	0.25
10% ammonium peroxodisulfate (w/v in H_2O)	0.125	0.125	0.125	0.125
TEMED	0.02	0.02	0.02	0.02

After addition of the catalyst TEMED the gel solution is poured vertically into a support consisting of a glass plate and a notched Teflon plate (10 x 8 cm each), that are separated by two 1.0 mm or 1.5 mm spacers at both sides. Between the upper margin of the Teflon plate and the upper end of the running gel a space of about 1.5 cm should be left free. Immediately after pouring the gel this space is filled with H_2O in order to obtain a horizontal and regular surface. As soon as the gel is polymerised (after about 30 min) the H_2O can be removed.
For preparation of the stacking gel the following reagents are then mixed in a beaker:

1.65 ml	acrylamide : N,N'-bisacrylamide (30% : 0.8%)
1.3 ml	1 M Tris-HCl pH 6.8
6.6 ml	H_2O
0.1 ml	10% SDS
0.05 ml	10% ammonium peroxodisulfate
0.008 ml	TEMED

The stacking gel is immediately poured into the remaining space between the running gel and the upper margin of the Teflon plate and a comb is inserted into the stacking gel, carefully avoiding formation of air bubbles. After complete polymerisation the comb can be removed and the complete SDS gel can be mounted into the electrophoresis apparatus. Tris-glycine buffer (250 mM glycine; 25 mM Tris base; 0.1% SDS; pH 8.3) is then added to the top and bottom reservoirs, the samples are loaded into the wells, and electrophoresis is started by applying a voltage of 8 V/cm (anode at the plug opposite the wells). When the bromophenol blue contained in the protein samples reaches the bottom of the gel the power supply can be turned off and the gel can be used for Coomassie blue staining or Western blotting.

20. Coomassie blue staining of SDS-polyacrylamide gels (Meyer and Lamberts, 1965)

After electrophoresis the SDS-polyacrylamide gel is soaked first for 1 hour in coomassie blue staining solution (0.2% coomassie brillant blue R 250; 45% ethanol; 10% acetic acid) and then for one or more hours in destain solution (45% ethanol; 10% acetic acid).

21. Immunoblot (Western Blot) analysis (Beier *et al*., 1998)

At the end of the electrophoresis run the gel device is removed from the electrophoresis apparatus, the Teflon plate is lifted off and the gel is covered with a nitrocellulose filter of the same size, that had been soaked in Tris-glycine buffer (192 mM glycine; 25 mM Tris base; 0.037% SDS; pH 8.3). The gel and its attached filter are then sandwiched between two moistened pieces of Whatman paper, two porous pads and two plastic supports. This construction is fixed with rubber bands and immersed in an electrophoresis tank that contains Tris-glycine buffer. An electric current of 200 mA is applied (anode at the pole next to the nitrocellulose filter) and proteins are transferred to the filter for 30 min. The blot support is then dismantled and successful transfer of proteins from the gel to the nitrocellulose filter is controlled by transient staining with Ponceau S solution. The filter is therefore incubated for 5-10 min with gentle agitation in a solution of 0.2% (w/v) Ponceau S; 3% (w/v) trichloroacetic acid; 3% (w/v) sulfosalicylic acid until protein bands become visible. The stain is then removed by extensive washing with H_2O and the filter can be used for immunological detection of proteins.

To block nonspecific binding sites on the nitrocellulose, the filter is therefore first incubated for 1 hour at room temperature with constant agitation in an excess volume of blocking solution (PBS containing 3% low-fat milk and 0.1% Triton X-100). One of the specific rabbit polyclonal antibodies is then added in an appropriate dilution (1:6000 for αGroEL and αCagA, 1:8000 for αUreAB and αVacA) to 10-20 ml fresh blocking solution and incubation is continued for 1 hour. After three washes of 5 min each in excess amounts of blocking solution filters are incubated for 1 hour with peroxidase-conjugated α–rabbit immunoglobulin (Sigma) that had been diluted 1:2000 in blocking solution. Filters are washed again as before and finally developed with an ECL™ chemiluminescence detection system (Amersham).

22. Analysis of radioactively labeled DNA fragments by urea polyacrylamide gel electrophoresis

Radioactively labeled DNA fragments are usually size-fractionated by electrophoresis in denaturing polyacrylamide gels. For electrophoresis of DNA fragments ranging between 60 and 250 bp a 6% gel is routinely used, smaller DNA fragments can be effectively separated by 8% gels or gels with even higher acrylamide concentrations.

Stock solutions containing the different acrylamide concentrations are first prepared by dissolving the following reagents in a final volume of 600 ml 1x TBE (90 mM Tris-HCl pH 8.3; 90 mM boric acid; 2.5 mM EDTA).

	6%	8%	10%	20%
urea	288 g	288 g	288 g	288 g
acrylamide	34 g	45.5 g	57 g	114 g
N,N'-bisacrylamide	1.8 g	2.3 g	3 g	6 g

The resulting acrylamide/N,N'-bisacrylamide solutions are stirred for 20 min in the presence of 20 g amberlit resin for deionisation, filtered through a disposable 0.2 µm filter, and then stored at room temperature in the dark until usage.

For preparation of a urea/polyacrylamide gel, a rectangular glass plate of 40 x 20 cm is placed exactly over a notched glass plate of the same size and two 0.35 mm spacers are inserted at both longitudinal sides in order to keep the glass plates apart. Fourty ml of the acrylamide/urea solution of the desired concentration is then mixed with 100 µl ammonium peroxodisulfate solution (10% w/v in H_2O) and 25 µl TEMED and poured immediately into the space between the glass plates. After that a comb is inserted into the gel solution and the two glass plates are fixed against each other with 4 to 6 clamps. When polymerisation has occurred, the comb is removed, the gel is mounted into the vertical electrophoresis chamber and the top and bottom reservoirs are filled with 1x TBE. The wells are extensively rinsed with 1x TBE to remove all remaining traces of urea and/or unpolymerised acrylamide, and after a short prerun (40 W; 1000 V; 20 min; anode at the plug opposite the wells), the samples can be loaded. Electrophoresis is carried out by applying a constant voltage (1400-1700 V; 40 W) and is terminated usually when the bromophenol blue contained in the samples reaches the bottom of the gel. The gel is then removed from the electrophoresis chamber, liberated from the glass plates and attached to a piece of Whatman 3MM paper. After being covered with Saran Wrap it is then dried in a gel-dryer for 1 hour at 80 °C and finally exposed to X-ray film.

23. RNA preparation (Scarlato *et al*., 1995)

Twenty-five ml of LB medium or modified Brucella broth are inoculated with 250 µl of an overnight culture of an *E. coli* or *H. pylori* strain, respectively, and incubated in a shaker at 37 °C. When the culture has reached an OD at 590 nm of 1.0, cells are harvested by centrifugation (8000 rpm; 10 min; 4 °C; JA20), resuspended in 3.7 ml of solution I (100 mM Tris-HCl pH 7.5; 2 mM Na_2EDTA; 1% SDS) and boiled for 5 min to allow lysis. After 5 min on ice cellular debris and chromosomal DNA are precipitated by the addition of 300 µl 1 M KCl and a further 10 min incubation on ice. Thereupon the sample is centrifuged (8000 rpm; 10 min; JA20) and 3.5 ml of the remaining supernatant is mixed with 4.56 g of CsCl. The RNA is then sedimented by centrifugation (35000 rpm; 15-20 hours; 17 °C; SW65), resuspended in 500 µl TE (10 mM Tris-HCl pH 8.0, 1 mM sodium EDTA), extracted once with an equal volume of phenol/chloroform (1:1) and

precipitated by the addition of 55 µl 10 M LiCl and 1 ml ethanol. After centrifugation the RNA is resuspended in 200 µl TE, quantitated by OD_{260}/OD_{280} readings, reprecipitated with 20 µl 10 M LiCl and 550 µl ethanol and stored at -20 °C.

24. 5'-labeling of oligonucleotides

In a final volume of 10 µl 3 pmol of oligonucleotide are mixed with 1 µl freshly prepared 10x kinase buffer (500 mM Tris-HCl pH 7.2; 100 mM $MgCl_2$; 50 mM DTT; 50 µg/ml BSA; 50 mM spermidine), 3 µl [γ-^{32}P]-ATP (5000 Ci mmol^{-1}) and 1 µl T4 polynucleotide kinase. The sample is incubated for 30 min at 37 °C and the labeled oligonucleotide is purified by centrifugation (700 g; 5 min) through a Chromaspin TE-10 column (Clontech) according to the supplier's instructions.

25. Primer extension analysis

0.4 - 1.0 pmol 5'-labeled oligonucleotide are coprecipitated with 15 µg of *E. coli* or *H. pylori* total RNA, dried, and resuspended in 5 µl H_2O, 2 µl 2 mM dNTPs and 2 µl 5x reverse transcription buffer (cDNA synthesis kit, Boehringer Mannheim). The reaction mixture is then incubated for 1 min at 95 °C, 1 µl of AMV-reverse transcriptase (20 U/µl; Boehringer Mannheim) is added, and reverse transcription is carried out by an incubation at 45 °C for 45 min. The sample is then incubated for 10 min at room temperature with 1 µl RNAse A (1 mg/ml) for RNA digestion, extracted once with an equal volume of phenol/chloroform (1:1), ethanol-precipitated and resuspended in 6 µl sequencing loading buffer (T7 sequencing^{TM} kit, Pharmacia). After denaturation at 95 °C for 2 min samples are subjected to 6% urea polyacrylamide gel electrophoresis. Gels are then dried in a gel-dryer and autoradiographed.

26. *In vitro* transcription

Three µg of plasmid DNA are incubated for 1 hour at 37 °C with 0.4 mM rNTPs and an appropriate amount of RNA polymerase in 50 µl transcription buffer (40 mM Tris-HCl pH 7.5; 6 mM $MgCl_2$; 2 mM spermidine; 5 mM DTT; 10 mM NaCl). The reaction mixture is then extracted once with an equal volume of phenol/chloroform (1:1), ethanol-precipitated and resuspended in 20 µl TE. Three µl are then used for primer extension experiments.

27. Labeling of DNA fragments at specific restriction sites

For footprinting or S1 nuclease mapping experiments DNA probes are usually required, that are radioactively labeled at only one of their two extremities. In order to prepare such a specifically labeled probe the fragment of interest is first cloned into a vector by the use of two different restriction enzymes, preferably enzymes that produce 5' overhangs after cleavage. 1.5 pmol of the resulting recombinant plasmid is then cleaved with one of the two restriction enzymes used for cloning, the two resulting 5'-phosphate ends are dephosphorylated with alkaline phosphatase, and the sample is phenol/chloroform (1:1) extracted and precipitated. Thereafter the digested plasmid DNA is resuspended in 5 µl H_2O and the two dephosphorylated 5' ends are labeled by the addition of 1 µl freshly prepared 10x PNK buffer (500 mM Tris-HCl pH 7.2; 100 mM $MgCl_2$; 50 mM DTT; 50 µg/ml BSA; 50 mM spermidine), 3 µl [γ-^{32}P]-ATP (5000 Ci mmol^{-1}) and 1 µl T4 polynucleotide kinase (10 U/µl). After an incubation of 30 min at 37 °C the second restriction enzyme of the two that had been used for cloning is added to the reaction with the appropriate buffer, thereby bringing the reaction to a final volume of 40-50 µl. Incubation is continued for at least 1 hour at 37 °C and the sample is then loaded on a 4-6% acrylamide gel (20:1 acrylamide:N-N'-bisacrylamide) and electrophoresed in 1x TBE at 150-200 V for 3-4 hours. The gel is exposed for 5-10 min to an autoradiography film, and the labeled fragment is localised with the help of the developed film and cut out of the gel with a razor blade. After that the gel slice containing the radioactive fragment is incubated over night at 37 °C under continuous shaking with 3 ml of elution buffer (10 mM Tris-HCl pH 8.0; 1 mM EDTA; 300 mM sodium acetate pH 5.2; 0.2% SDS). On the next day, the sample is extracted once with an equal amount of phenol/chloroform (1:1) for removal of remaining gel pieces and precipitated by the addition of 2 volumes ethanol. The labeled fragment is finally resuspended in 100 µl of TE and is then ready for use in S1 nuclease mapping or footprinting experiments.

28. S1 nuclease mapping (Scarlato *et al*., 1990)

Approximately 20 fmol of specifically 5'-end labeled fragment is coprecipitated with 15 µg of total RNA and resuspended in 20 µl of hybridisation buffer (80% formamide; 60 mM Tris-HCl pH 7.5; 400 mM NaCl; 0.4 mM EDTA). The mixture is overlayed with 5 µl mineral oil, denaturated at 100 °C for 3 min and then incubated at an annealing temperature (T_m) calculated on basis of the following formula: $T_m = 81.5 + 0.5(\%GC) + 16.6 \lg[Na+] - 0.6 (\%formamide)$. After 4 to 16 hours of incubation, 180 µl of ice-cold S1 buffer (33 mM sodium acetate pH 5.2; 5 mM $ZnSO_4$; 250 mM NaCl) and 1 µl S1 Nuclease (400 U/µl) are added and S1 digestion is carried out for 30 min at 37 °C. Samples are then extracted once with an equal volume of phenol/chloroform (1:1), ethanol-precipitated and resuspended in 5 µl of sequencing loading buffer (T7 sequencing™ kit,

Pharmacia). After denaturation at 100 °C for 2 min, samples are subjected to 6% or 8% urea polyacrylamide gel electrophoresis and autoradiographed.

29. Sequencing of cloned DNA fragments

Cloned DNA fragments are sequenced according to the dideoxy chain termination method (Sanger *et al.*, 1977; Tabor and Richardson, 1987) using [α-^{33}P] dATP (Amersham) and a T7 Sequencing^{TM} kit (Pharmacia) or by using an Applied Biosystems 373 automated DNA sequencer.

30. End labeling of DNA fragments with klenow enzyme (Sambrook *et al*., 1989)

DNA fragments with 5' overhangs that contain T or G nucleotides can be efficiently radiolabeled by 3' incorporation of labeled ATP and/or CTP. One µl of the DNA fragment (1 pmol/µl) is therefore mixed with 5 µl 10x klenow buffer (500 mM Tris-HCl pH 7.6; 100 mM $MgCl_2$), 2.5 µl 5 mM dGTP, 2.5 µl 5 mM dTTP, 1.5 µl [α-^{32}P]-dATP (3000 Ci/mM), 1.5 µl [α-^{32}P]-dCTP (3000 Ci/mM), 35 µl H_2O and 1 µl klenow enzyme (5 U/µl). After 30 min incubation at room temperature the labeled fragment can be purified by centrifugation (500 g; 5 min) through a Chromaspin TE-10 column (Clontech) according to the supplier's instructions.

31. Electrophoretic mobility shift assay (Zu *et al*., 1996)

To determine if a given protein preparation binds specifically to a particular DNA fragment, an electrophoretic mobility shift assay is usually performed. This assay makes use of the fact that protein-DNA complexes migrate through polyacrylamide gels more slowly than unbound DNA fragments, and is therefore a rapid and easy method to study the DNA binding activity of proteins.

For the binding reaction approximately 10,000 cpm (ca. 10 fmol) of the radioactively labeled fragment is mixed with different amounts of the protein of interest in 20 µl of binding buffer (10 mM Tris-HCl pH 8.0; 50 mM NaCl; 10 mM $MgCl_2$; 1 mM DTT; 0.1% NP-40). After 15 min incubation at room temperature, samples are loaded on a 4% (20:1 acrylamide:N,N'-bisacrylamide) or 6% (60:1 acrylamide:N,N'-bisacrylamide) gel, and electrophoresed in 0.25x TBE buffer (22.5 mM Tris-HCl pH 8.3; 22.5 mM boric acid; 0.6 mM EDTA) at 150-200 V for 2-3 hours. Gels are then dried and exposed to film for autoradiography.

32. DNase I footprinting (Zu *et al*., 1996)

Specific binding sites of a protein on a given DNA sequence can be visualised by the method of DNase I footprinting. The DNA fragment, which has been radiolabeled at one of its two ends, is therefore incubated with the protein of interest and is then digested with DNase I under such conditions that lead to an average of one cleavage per DNA molecule. All bases of the fragment are attacked rather randomly in this way, with the exception of those bases that are covered by the DNA binding protein and are therefore not accessible to the enzyme. Consequently, the mixture of DNA fragments that results from the digestion does not contain the fragments that correspond to cleavage at these bases. The separation of the reaction mixture on a denaturing polyacrylamide gel therefore reveals the sites of DNA-protein interactions as gaps in the digestion pattern.

For formation of the protein-DNA complexes approximately 10,000 cpm (ca. 10 fmol) of the radiolabeled fragment is mixed with different amounts of the protein of interest in 50 µl of footprinting buffer (10 mM Tris-HCl pH 8.0; 50 mM NaCl; 10 mM KCl; 1 mM DTT; 0.1% NP-40; 10% glycerol). After 15-20 min incubation at room temperature 1 µl microliter of DNase I (0.002-0.02 U/µl depending on the DNA fragment) in footprinting buffer containing 5 mM $CaCl_2$ is added and incubation is continued for 1 min at room temperature. DNase I digestion is then stopped by addition of stop buffer (192 mM Na-acetate; 32 mM EDTA; 0,14% SDS; 64 µg/ml sonicated salmon sperm DNA). Samples are extracted once with 1 volume of phenol/chloroform (1:1), ethanol-precipitated and resuspended in 5 µl of sequencing loading buffer (T7 sequencing^{TM} kit, Pharmacia). After denaturation at 95 °C for 4 min, samples are subjected to 6% or 8% urea gel electrophoresis and autoradiographed.

33. G+A specific cleavage of end labeled DNA fragments (Maxam and Gilbert, 1980)

In most footprinting experiments a size marker for the DNase I digested labeled fragments is required in order to localise possible sites of protein-DNA interactions. Therefore a G+A sequencing reaction of the DNA fragment employed in the footprinting experiment is usually run in parallel with the DNase I digestion.

For the G+A specific cleavage 20-50 fmol of the terminally labeled DNA fragment are made up to a volume of 8 µl with TE, mixed with 1 µg poly-d(IC) and 1 µl 50% formic acid and incubated at 37 °C for 6 min. One hundred and fifty µl of 1 M piperidine are then added to the reaction and incubation is continued at 90 °C for 30 min. After 5 min on ice the sample is mixed with 3 µl tRNA (1 µg/µl) and 1 ml n-butanol and the cleaved DNA is collected by centrifugation (10 min; 4 °C; 12000 rpm). The supernatant is removed and the DNA is mixed thoroughly with 150 µl 10% SDS and 1 ml n-butanol, recentrifuged (10 min; 4 °C; 12000 rpm), washed once with n-

butanol and then dried for 10-15 min in the speed-vac. Finally the DNA is resuspended in sequencing loading buffer (T7 sequencing^{TM} kit, Pharmacia), boiled for 4 min and loaded on a denaturing polyacrylamide gel together with the footprinting reaction.