PROTOCOL 1: EXTRACTION OF DNA FROM BACTERIA

The basic "standard" procedures for isolation of bacterial DNA are based on lysozyme digestion of the cell wall, detergent lysis, disruption of protein-nucleic acid complexes and phenol:chlorophorm extraction to remove proteins. A number of other methods have come into use; these are based on the chaotropic agent guanidine hydrochloride, and is often successful for species resistant to detergent lysis, for instance mycobacteria. The physiological state of cells sometimes affects the ease with which DNA can be extracted, either by making them difficult to lyse, or by producing large amounts of capsule or glycocalyx material, which is largely composed of polysaccharide. Since extracellular materials are produced mostly in physiologically limiting conditions, a sometimes successful solution is to work with very young cultures, even though large volumes may be required for harvesting enough cells.

The boiling method is rapid and renders DNA of a minimum purity but enough to used in PCR reactions.

The methods given here usually start from liquid cultures; most can also be used with cells cultured on solid media, by scraping them off and resuspending them in the appropriate buffer. Production of extracellular material is more likely to be a problem than with cells grown in liquid culture.

1.1. BOILING METHOD

Equipment

- Bacterial isolate cultured on an agar plate
- Inoculating loop
- Incubator
- Eppendorf tubes (vol 1,5 ml), sterilised
- Pipettes and sterilised tips
- Block heater (up to 100ºC)
- Centrifuge

Solutions

- Molecular grade water, autoclaved

Procedure

Add 100 µl of molecular grade water into an Eppendorf tube. Then, pick up 5 colonies from the agar plate and dissolve them in the Eppendorf tube.

1. Incubate at 100ºC for 10 minutes.
2. Add 900 µl of molecular grade water and mix well until the solution is homogeneous.
3. Harvest by centrifugation (12.000 rpm for 10 min)
4. Transfer supernatant to an sterile Eppendorf tube

For PCR applications, use 1-5 µl of the supernatant .

1.2. CHROMOSOMAL DNA EXTRACTION

Materials

- Bacterial isolate cultured on an agar plate
- Inoculation loop
- Incubator
- Eppendorf tubes (vol 1,5 ml), sterilised
- Pipettes and sterilised tips
- Centrifuge

Solutions

- Chloroform: iso-amyl alcohol (24:1)

- Phenol/chloroform: iso-amyl alcohol (1:1 v/v)

- CTAB/NaCl: Mix 10% Hexadecyltrimethylammonium Bromide and 5M NaCl 5M (1:1). Store at room temperature.

- RNAse (10 mg/ ml):Disolve 100 mg RNAse in 10 ml sterile distilled water and incubate at 90 ºC for 10 minutes to inactivate DNAss. Keep at – 20 ºC until use.

- Proteinase K (20 mg/ ml): Disolve 100 mg proteinase K in 5 ml sterile distilled water. Keep at – 20 ºC.

- TE (pH 8.0): 10 mM Tris-HCl**,** 1mM EDTA. Autoclave and keep at room temperature.

- 10% SDS (w/v): Disolve 10 g of SDS in 100 ml of distilled water. Keep at room temperature.

- 5M NaCl: Disolve 292.2 g of NaCl in a final volume of 1 litre. Autoclave.

- Molecular biology grade water, autoclaved

Procedure

1. Resuspend a loop of colonies from a cultured agar plate in 500 µl of TE and dissolve completely.
2. Add 30 µl of 10% SDS. Mix well.

3. Add 3 µl of proteinase K (20 mg/ml). Incubate at 37 ºC for 60 minutes.
4. Add first 100 µl of 5M NaCl, mix well and then, 80 µl of CTAB/NaCl. Mix by inverting the tube (gentle tilting) and incubate at 65ºC for 10 min.
5. To the solution add an equal volumen of phenol/chloroform:iso-amyl alcohol (25/24:1), mix to emulsify and spin for 5 minutes at 8000 rpm. Pipette upper layer into a fresh tube..
6. Repeat step 5
7. Add RNAse to a final concentration of 50 µg/ml and incubate for 30 minutes at 37 ºC
8. Repeat step 5
9. Precipitate DNA adding 1/10 vols of 5M NaCl and 2 vols of 100% cold ethanol and keep at -20º C o/n or at -80º C for 60 minutes.
10. Spin at 12.000 rpm for 20 minutes.
11. Remove supernatant.
12. Wash by adding 70% etanol followed by centrifugation as in step 10. Dry completely.
13. Redissolve in 50-200 µl of TE and keep at -20 ºC

Guidelines for quantification of the DNA are described in the section of "Basic procedures" of the course.

1.3. EXTRACTION OF PLASMID DNA USING THE "Plasmid Mini Kit" (Qiagen)

Materials

- Bacterial isolate cultured in liquid media
- Inoculating loop
- Incubator
- Eppendorf tubes (vol 1,5 ml), sterilised
- Pipettes and sterilised tips
- Block heater (up to 100ºC)
- Centrifuge
- "Plasmid Mini Kit" (Qiagen)

Solutions

- Molecular grade water, autoclaved
- Saline solution
- TES**:** 0.01M Tris (pH7.8), 0.05M EDTA, 0,5%SDS
- Proteinase K (stock, 50mg/ml in distilled water)
- TE: 10 mM Tris–Cl (pH 8.0); 1mM EDTA

Procedure

1. Inoculation of bacteria.

1.1.Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 10 ml liquid medium and incubate at 37° C o/n (best if it is in an orbital incubator).

2. Harvesting the cells.

2.1. Spin at 10000 rpm for10 min and discard supernatant.

2.2. (adapted for *A. baumannii*) Wash the pellet with 5 ml of saline solution and spin at 10000 rpm for 10 min. Discard supernatant and repeat .

3. Treatment with TES and proteinase K. (adapted for *A. baumannii*)

3.1. Add 1 ml of TES and resuspend the pellet. Then add 0.5 µl of proteinase K and incubate at 37° C for 30 min.Centrifuge at 12000 rpm for 10 min and discard supernatant.

4. Plasmid Mini Kit, Qiagen .

4.1. Resuspend bacterial pellet in 300µl buffer P1*.

4.2. Add 300µl buffer P2*, mix gently but thoroughly by inverting the tube 4–6 times and incubate at room temperature for 5 min.

4.3. Add 300µl of chilled buffer P3*, mix inmediatly but gently by inverting the tube 4–6 times and incubate on ice for 5 min.

4.4. Centrifuge at 10000–13000 rpm, for 10 min and transfer supernatant containing plasmid promptly to an sterile Eppendorf tube. Repeat once.

4.5. Equilibrate a Quiagen tip with 1 ml buffer QBT* and allow the columna to empty by gravity flow.

4.6. Apply supernatant to the tip and allow it to enter the resin by gravity flow.

4.7. Wash with 2 x 2ml buffer QC*.

4.8. Elute DNA with 800µl buffer QF*.

4.9. Precipitate DNA by adding 0.7 volumes room-temperature isopropanol to the eluted DNA. Mix and centrifuge inmediatly at 10000 rpm for 30 min. Carefully decant the supernatant.

4.10. Wash DNA pellet with 1 ml of room-temperature 70% ethanol, and centrifuge at 10000 rpm for 10 min. Carefully decant the supernatant.

4.11. Air-dry the pellet and redissolve in a suitable volumen of buffer.

***Procedure from the Qiagen Plasmid Purification Handbook, Qiagen Ltd, UK.**

Buffer	Composition	Storage
P1	50 mM Tris–Cl, pH 8.0; 10 mM EDTA; 100µg/ml RNase A	2–8°C, alter adding RNase A
P2	200 mM NaOH, 1%SDS(w/v)	15–25°C
P3	3.0M Potasium acetate pH 5.5	15–25°C or 2–8°C
QBT	750mM NaCl; 50mM MOPS, pH 7.0 ; 15% isopropanol(v/v); 0,15% Triton X–100(v/v)	15–25°C
QC	1.0 mM NaCl; 50mM MOPS, pH 7.0; 15% isopropanol(v/v)	15–25°C
QF	1.25 M NaCl; 50mM Tris–Cl, pH 8.5;15% isopropanol(v/v)	Store at 15–25°C but must be at 65°C when used.
TE	10 mM Tris–Cl, pH 8.0; 1mM EDTA	15–25°C