CORYNEBACTERIUM DIPHTHERIAE RIBOTYPING METHOD

This protocol is divided into the following sections:

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Based on: Grimont et al (2004) & Regnault et al (1997)

1. Extraction and Restriction Endonuclease Digestion of *Citrobacter koseri*

SUMMARY
This section describes the method for the extraction of and restriction endonuclease digestion of *C. koseri* DNA. In the ribotyping of *Corynebacterium diphtheriae, C. koseri* DNA digested by *Mlu*I is used as a molecular marker.

SAFETY
DNA extraction using phenol/chloroform MUST be carried out in a Class I Safety Cabinet at all times and NOT on the open bench. Nitrile gloves and safety glasses should be worn.
Phenol is highly corrosive. Phenol burns, in the event of contact with skin, eyes, etc wash with water, cover with glycerol and seek medical advice immediately.
Chloroform must not be handled by pregnant workers.

1.0 MATERIALS

1.1. Nutrient agar plates
1.2. Lysis buffer
1.3. 20% SDS
1.4. Proteinase K
1.5. Phenol/Chloroform
1.6. Chloroform
1.7. 100% ethanol
1.8. TE Buffer
1.9. Sterile distilled water
1.10. Nuclease free water
1.11. *Mlu*I restriction enzyme
1.12. *Mlu*I 10x restriction buffer
1.13. 10% Ficoll Bromophenol Blue
1.14. Waterbath or heat block
1.15. Shaker
1.16. Whirlimixer
1.17. Spiromixer
1.18. Centrifuge
1.19. GeneQuant Spectrophotometer
1.20. 100ml conical flasks
1.21. 2ml eppendof tubes
1.22. 1ml, 10ml, 20ml and 100ml pipettes
1.23. Pipette tips
1.24. Sterile disposable loops

METHOD

2.0 Extraction of DNA

DAY ONE

2.1. The reference strain of *Citrobacter koseri* used as a molecular marker in the ribotyping of *Corynebacterium diphtheriae* is stored at -20°C in glycerol blood broths. Take out the strain from the freezer in a suitable container/rack and transfer it to a Class I Safety Cabinet.

2.2. Gently scrape off the surface of the frozen glycerol broth with a disposable loop and inoculate ten nutrient agar plates in the Class I Safety Cabinet wearing gloves and safety glasses.

2.3. Return the frozen glycerol broth to the -20°C freezer after use immediately. Do not let it thaw out completely. Incubate the plates overnight at 37°C.

DAY TWO

2.4. In the Class I Safety Cabinet wearing gloves and safety glasses, remove the growth from the agar plates using a sterile disposable loop and suspend in 30ml of lysis buffer in a 100ml conical flask. This should be a very turbid suspension.

2.5. Add 650µl of 20% SDS and mix well. One should see the suspension change consistency.

2.6. In a fume cupboard wearing nitrile gloves and safety glasses, prepare 150µl of Proteinase K in lysis buffer (Proteinase K 25mg/ml - stored at -20°C).

2.7. Add 150µl of Proteinase K into the flask and leave overnight at 37°C with gentle shaking. The solution should become homogeneously viscous. Do not shear the chromosomal DNA.

DAY THREE

2.8. Aliquot 15mls of the bacterial lysate into two separate plastic tubes (resistant to phenol/chloroform).

2.9. In a Class I Safety Cabinet wearing nitrile gloves and safety glasses, add 15ml of phenol/chloroform into each tube and mix thoroughly but gently to obtain a milky emulsion.

2.10. Centrifuge at 4000g for ten minutes. One should be able to see two separate layers. Remove the upper aqueous layer (supernatant) in each tube and transfer into conical flasks.

2.11. Pour in two volumes of cold 100% ethanol into each flask. The DNA, which forms a dense stringy precipitate within a few seconds should be removed using a pipette tip and place in a fresh tube containing 5ml of TE buffer. Repeat procedure for the second flask.

2.12. To resuspend the DNA in the TE buffer, rock gently overnight at room temperature.

DAY FOUR

2.13. Aliquot the extracted DNA solution into 2ml eppendof tubes.

2.14. Store at -20°C.

3.0 Determining the Nucleic Acid Concentration

Gloves and safety glasses must be worn during the measurement of nucleic acid concentration. If used, quartz capillaries are fragile and may break unless handled with care. Capillaries MUST be discarded into the sharps bin.

3.1. For good enzyme restriction the DNA has to be of high quality, pure and without protein contamination. If measured, the absorbance ratio 260nm/280nm should be about 1.8-1.9. Ratios less than <1.7 indicate significant protein contamination.

3.2. In the ribotyping of *Corynebacterium diphtheriae*, *C. koseri* DNA digested by *Mlu*I is used as a molecular marker. For a 20-well gel, at least four molecular marker lanes should be included. Each *C. koseri* digest should contain 5µg of DNA in a volume of 20µl. To determine for each sample how many uls is equal to 5ug, divide 5 by the concentration of DNA obtained and this will give you the number of uls required. (For example, if the concentration is 0.5, then divide 5 by 0.5, which is equal to 10. In this case, this means that 10ul of the sample is equal to 5µg DNA.) Determine the volume needed for each sample.

3.3. Label one fresh eppendof tube for each ribotyping marker lane and pipette the amount of DNA required which is equal to 5µg of DNA.

3.4. Add the necessary amount of sterile distilled water to make up the volume to 20µl.

4.0 Cleavage Of The DNA With Restriction Endonuclease *Mlu*I

Cleavage of DNA should be performed in accordance with the manufacturers instructions, which are supplied with the restriction enzyme. Gloves and safety glasses must be worn.

4.1. In each tube add 2.5µl of 10x *Mlu*I restriction buffer and 1.0µl of *Mlu*I restriction enzyme. The enzyme and buffer should be kept on ice at all times and when the enzyme is added to the tubes containing the DNA, they also should be kept on ice. Never allow the enzyme concentration to be greater than 10% (2.35µl) as the glycerol in the storage buffer affects the enzyme activity.

4.2. The tubes should be closed and whirlimixed for a couple of seconds and then centrifuged for a few seconds at 10,000 rpm.

4.3. The tubes should then be incubated at 37°C for four hours in a waterbath or on a heat block.

4.4. Add 5µl of 10% Ficoll Bromophenol Blue to the tubes and store at 4°C.

2. Extraction and Restriction Endonuclease Digestion of *Corynebacterium diphtheriae*

SUMMARY

This section describes the method for the extraction of and restriction endonuclease digestion of *C. diphtheriae* DNA. In order to get reproducible restriction profiles, the DNA has to be of high molecular weight and free of any contaminants that might inhibit the activity of the restriction enzyme. The bacterial culture has to be in the late of the exponential phase and different procedures (mechanical, chemical and enzymatic) are applied in order to achieve cellular lysis. The cellular debris is eliminated by centrifugation, the proteins are precipitated out by ammonium acetate and chloroform/isoamylalcohol, and the extracted DNA is washed in alcohol. The DNA in solution is then digested with a restriction enzyme.

SAFETY

The initial harvesting and extraction which involves handling live cultures of *C. diphtheriae* must be carried out in a Class I Safety Cabinet at all times and NOT on the open bench. Gloves and safety glasses should be worn. This work should only be undertaken in a restricted access laboratory, by designated members of staff with known current diphtheria immunity and not by pregnant workers.

1.0 MATERIALS

1.1. Columbia blood agar (CBA) plates
1.2. Lysozyme
1.3. Lysis buffer
1.4. Proteinase K
1.5. GES reagent
1.6. 7.5M Ammonium acetate
1.7. Chloroform/isoamylalcohol (24:1)
1.8. Isopropanol
1.9. 70% ethanol
1.10. Sterile distilled water
1.11. Nuclease free water
1.12. *Bst*EII restriction enzyme
1.13. *Bst*EII 10x restriction buffer
1.14. 10% Ficoll Bromophenol Blue
1.15. Water bath or heating block
1.16. Whirlimixer
1.17. Spiromixer
1.18. Centrifuge
1.19. GeneQuant Spectrophotometer
1.20. 2ml eppendorf tubes with lockable tubes
1.21. Sterile disposable loops

METHOD

2.0 Extraction of DNA

DAY ONE

2.1. All reference strains and routine isolates are stored at -20°C in glycerol blood broths. Take out the vials of strains from the freezer in a suitable container/rack and transfer them to a Class I Safety Cabinet.

2.2. For each *C. diphtheriae* strain to be ribotyped, gently scrape off the surface of the frozen glycerol broth with a disposable loop and inoculate one CBA plate in the Class I Safety Cabinet wearing gloves and safety glasses.

2.3. Return the frozen glycerol broths to the -20°C freezer after use immediately. Do not let them thaw out completely. Incubate the CBA plates overnight at 37°C.

DAY TWO

2.4. From these pure cultures, inoculate two further CBA plates as lawns for each of the test isolates in the Class I Safety Cabinet wearing gloves and safety glasses. Incubate for 16 to 24 hours at 37°C.

DAY THREE

2.5. In a fume cupboard wearing nitrile gloves and safety glasses, prepare 50 mg/ml of lysozyme in distilled water (Lysozyme is stored at -20°C). The amount required is 200µl per strain to be ribotyped.

2.6. Dispense 200µl lysozyme per DNA extraction into 2ml eppendorf tubes.

2.7. In the Class I Safety Cabinet wearing gloves and safety glasses, remove the growth from the CBA plates with the lawn cultures using a sterile disposable loop and suspend in the lysozyme. Close the caps of the tubes and vortex the contents of the tubes (the whirlimixer should be placed inside a Class I Safety Cabinet when vortexing). Incubate tubes at 37° for three hours.

2.8. In a fume cupboard wearing nitrile gloves and safety glasses, prepare 25mg/ml of Proteinase K in lysis buffer (Proteinase K is stored at -20°C). The amount required is 10µl per strain to be ribotyped.

2.9. Place tubes inside the Class I Safety cabinet and add 10µl of Proteinase K into each eppendorf tube. Vortex as before and incubate in a 50°C water bath/heating block for one hour.

2.10. Place tubes inside the Class I Safety cabinet and add 500µl of GES reagent into each tube. Vortex as before and place tubes on a spiramix for 15min.

2.11. Place tubes inside the Class I Safety cabinet and add 250µl of cold 7.5M ammonium acetate and 250µl of chloroform/isoamyl alcohol (24:1) into each tube. Vortex as before and leave on ice for ten minutes. Centrifuge at 14,000 rpm for ten minutes in a sealed centrifuge rotor. When taking tubes out of the centrifuge, remove the centrifuge rotor and place it inside the Class I Safety cabinet before removing lid of the rotor.

2.12. There should be two separate layers visible. Remove the upper aqueous layer (supernatant) in each tube using a 1ml pipette and transfer to a fresh tube and add 0.54 volumes of isopropanol.

2.13. Precipitate out the DNA by closing the tube and inverting it up and down several times. The DNA, which forms a dense stringy precipitate within a few seconds, should be removed using a pipette tip and placed in a fresh tube containing 1ml of 70% ethanol. Invert the tube up and down to wash the DNA. Then remove the ethanol with a pipette.

2.14. Wash the DNA again as before by adding 1ml fresh 70% ethanol into the tube.

2.15. Aspirate off the ethanol and dry the DNA at 37°C (ten to twenty minutes is usually sufficient).

2.16. Reconstitute the DNA in 100µl of nuclease free water (50µl if there is only a small amount of DNA). Incubate overnight at 4°C to dissolve the DNA.

2.17. Store at -25°C.

3.0 Determining the Nucleic Acid Concentration

Gloves and safety glasses must be worn during the measurement of nucleic acid concentration. If used, quartz capillaries are fragile and may break unless handled with care. Capillaries MUST be discarded into the sharps bin.

3.1 For good enzyme restriction the DNA has to be of high quality, pure and without protein contamination. If measured, the absorbance ratio 260nm/280nm should be about 1.8-1.9. Ratios less than <1.7 indicate significant protein contamination.

3.2 Each digest should contain 5µg of DNA in a volume of 20µl. To determine how many µls is equal to 5ug, divide 5 by the concentration of DNA obtained and this will give you the number of µls required. For example, if the concentration is 0.5, then divide 5 by 0.5, which is equal to 10. In this case, this means that 10µl of the sample is equal to 5µg DNA. Determine the volume needed for each sample.

3.3 Label one fresh 1.5ml eppendorf tube for each sample and pipette the amount of DNA required which is equal to 5µg of DNA.

3.4 Add the necessary amount of sterile distilled water to make up the volume to 20µl.

4 Cleavage of the DNA with Restriction Endonuclease *Bst*EII

Cleavage of DNA should be performed in accordance with the manufacturers' instructions, which are supplied with the restriction enzyme. Gloves and safety glasses must be worn.

4.1 In each tube add 2.5µl of 10x *Bst*EII restriction buffer and 1.25µl of *Bst*EII restriction enzyme. The enzyme and buffer should be kept on ice at all times and when the enzyme is added to the tubes containing the DNA; they also should be kept on ice. Never allow the enzyme concentration to be greater than 10% (2.375µl) as the glycerol in the storage buffer affects the enzyme activity.

4.2 The tubes should be closed and whirlimixed for a couple of seconds and then centrifuged for a few seconds at 10,000 rpm.

4.3 The tubes should then be incubated at 60°C for four hours in a waterbath or on a heat block.

4.4 Add 5µl of 10% Ficoll Bromophenol Blue to the tubes and store at 4°C.

3. Agarose Gel Electrophoresis and Southern Blotting of *Corynebacterium diphtheriae* Restriction Fragments

SUMMARY

This section describes the method for agarose gel electrophoresis of *Corynebacterium diphtheriae* DNA restriction fragments. The DNA on the gel is visualised by staining the gel with ethidium bromide. Ethidium bromide binds to DNA and, when the gel is placed in a UV transilluminator, the DNA can be seen.

This document also describes the way in which DNA restriction fragments from agarose gels are transferred to a nylon membrane by a method known as Southern blotting and the DNA is then fixed onto the membrane using UV light.

SAFETY

Nitrile/latex gloves must be worn at all times. Ethidium bromide is a carcinogen. This procedure must NOT to be performed by pregnant workers. When using UV light always protect your eyes and skin by wearing a UV face shield and gloves.

1.0 MATERIALS

1.1. *C. diphtheriae* DNA samples digested with *Bst*EII restriction enzyme
1.2. *Citrobacter koseri* 32 DNA digested with *Mlu*I restriction enzyme
1.3. Agarose (Invitrogen)
1.4. TBE
1.5. Ethidium bromide
1.6. Denaturing solution
1.7. Transfer buffer
1.8. 2x SSC
1.9. 10% Ficoll Bromophenol Blue loading dye
1.10. Sterile distilled water
1.11. Electrophoresis tank (Pharmacia LKB GNA 200) or suitable tank
1.12. Gel tray and comb (Pharmacia)
1.13. Power pack
1.14. Levelling kit (horizontal table and spirit level)
1.15. Autoclave tape
1.16. Conical flask or suitable container
1.17. Measuring cylinder
1.18. Waterbath
1.19. Heated magnetic stirrers/fleas
1.20. Plastic sandwich boxes, one with a lid
1.21. Vacublot apparatus and pump
1.22. UV face shield
1.23. UV transilluminator
1.24. Nylon membrane (Hybond-N)

1.25. Pipettes
1.26. Filter paper
1.27. Saran wrap

METHOD

2.0 Preparation of agarose gel

2.1. Wash and seal the ends of a clean dry plastic gel tray supplied with the electrophoresis apparatus with autoclave tape so as to form a mould. Place the mould on the levelling table and level using the spirit level. Position the comb at one end of the gel tray. It should be 0.5-1.0mm above the base of the gel tray. The combs should make wells of 1mm wide. Do not use 2mm combs as the bands would be too diffuse.

2.2. Prepare sufficient electophoresis buffer (1x TBE) to fill the tank and for the gel itself (SOP R-6435). Add the correct amount of agarose to a measured quantity of 1x TBE in a conical flask or suitable container. To separate DNA restriction fragments of *C. diphtheriae* from 0.7 to 20kb, use a 0.8% agarose gel. For a large gel of 22 wells, add 200ml of 1x TBE to 2g of agarose in a conical flask.

2.3. Heat the suspension on a heated block with a magnetic stirrer for the minimum time required allowing all the grains of agarose to dissolve.

The agarose solution may boil violently if it has been heated for too long

2.4. Cool the solution to 55°C in a waterbath and pour the agarose into the gel tray, ensure there are no air bubbles trapped in the gel and leave to set for approximately 30 minutes. The gel is set when the agarose becomes translucent. The gel should be 4mm to 5mm thick.

2.5. When the gel is completely set, completely remove the comb and autoclave tape and mount the gel in the tray in the electrophoresis tank. Pour just enough 0.5x TBE buffer to cover the gel to a depth of about 5mm.

2.6. Add 5µl of 10% Ficoll Bromophenol Blue loading dye to each *C. diphtheriae* DNA sample and to each 20µl marker sample of *Citrobacter koseri*. For a 20-well gel, there should be at least four marker lanes.

2.7. Using a 1ml pipette, load each sample slowly into a separate well. The wells with the marker samples of *Citrobacter koseri* should be evenly spaced in the gel (one at each end and the others at regular intervals across the gel).

2.8. When all the samples are loaded, close the lid of gel tank and attach the electrical leads so that the DNA will migrate towards the anode (red lead). Apply a voltage of 30V, ensure the current is 50-60mA, and run the gel for 16 hours. If the leads have been attached correctly, bubbles should be generated at the anode and the cathode and, within a few minutes, the loading dye in the samples should migrate from the wells into the body of the gel.

CAUTION: Ethidium bromide is a carcinogen. This procedure must NOT be performed by pregnant workers. When using UV light always protect your eyes and skin by wearing a UV face shield and gloves.

2.9. After 16 hours, turn off the electric current and remove the leads and lid from the gel tank. Wear gloves and take the gel out of the tank very carefully and stain the gel with 1µg/ml ethidium bromide for 30 minutes in a plastic sandwich tray with a lid. Destain with sterile distilled water in a sandwich tray for 10 minutes and then the bands should be visualised with the UV transilluminator (254nm) to confirm the fragments have migrated as expected and photographed.

3.0 Southern blotting using a Vacugene

CAUTION: Gloves must be worn during this procedure to avoid contamination.

3.1. The vacuum blotting unit should be clean, dry and ready for use, with the vacuum pump and in-line liquid trap connected. Ensure that the rubber-sealing gasket is clean and dry.

3.2. Wet the porous support screen in distilled water and place it on the inner rim of the base unit with the shiny side up.

3.3. Place the plastic mask with the window on the support screen.

Note: The window on the plastic mask has to be cut beforehand. The window has to be cut so that it is at least 2cm smaller than the size of the gel on all four sides so that when the gel is placed on top of the plastic mask, the gel overlaps the mask.

3.4. Wearing gloves cut a piece of nylon membrane of the same size as the gel. Touch the membrane only at the very edge.

3.5. Take a plastic sandwich box that is slightly bigger than the membrane. Soak the membrane in 100ml of 2x SSC solution and ensure it is thoroughly wetted. The timing is not important in this case. Keep the 2x SSC for use later.

3.6. Wet the screen of the vacuum blotter using sterile distilled water and place on the inner rim of the base unit.

3.7. Place the membrane on the screen and overlay with the plastic mask. The window of the plastic mask should be slightly smaller than the membrane. The mask ensures the full effect of the vacuum is concentrated on the gel.

3.8. Position the pretreated nylon membrane under the mask so that it covers the mask's window completely, ensuring no air bubbles are trapped in between.

3.9. Denature the gel for 30 minutes in 250ml denaturing solution and rinse in sterile distilled water.

3.10. Starting with one of the gel edges, gradually slide the gel onto the membrane to fill the window. Avoid trapping air bubbles between the gel and the membrane.

3.11. Fit the top frame of the apparatus and secure it.

3.12. Connect the pump to the vacuum blotter and switch on the pump and adjust the suction to 45.

3.13. Apply 2 x 50ml of transfer buffer to the surface of the gel. Allow to dry (approximately 30 minutes) before adding another 50ml of transfer buffer.

3.14. After transfer, remove the gel and switch off pump. Mark the membrane with a `T' for top, to indicate the side of the membrane the DNA is on.

3.15. Rinse the membrane in the 2x SSC retained from earlier for two minutes.

3.16. Place the membrane between two pieces of filter paper and let it dry at room temperature.

4.0 Fixing DNA onto the nylon membrane using ultraviolet light

Caution: When using UV light always protect your eyes and skin by wearing a UV face shield and gloves.

4.1. Once the nylon membrane has dried, wrap in Saran Wrap transparent cling film and place, DNA side down, on a UV transilluminator.

4.2. Switch the UV light on and leave the membrane exposed for 30 seconds.

4.3. After 30 seconds switch off the transilluminator and store the membrane in a safe dark and dry place until the final stage of the ribotyping procedure, hybridisation with probes is carried out. The membrane may be stored indefinitely.

4.4. Screens should be rinsed thoroughly washed and left to dry .The vacuum blotter should also be washed thoroughly with water.

4. Prehybridisation, Hybridisation and Detection of Hybrids used in the Ribotyping of *Corynebacterium diphtheriae*

SUMMARY
This section describes the way membrane bound *C. diphtheriae* DNA digests are prehybridised and then hybridised using Oligomix5 probe in a hybridisation oven and/or waterbath. It also describes immunological detection of hybrids.

SAFETY
Nitrile/Latex gloves must be worn at all times.

1.0 MATERIALS

1.1. Wash solution A
1.2. Wash solution B
1.3. Buffer 1
1.4. Buffer 2
1.5. Buffer 3
1.6. DIG easy hybridisation solution
1.7. Anti-DIG AP-conjugated
1.8. Nitro blue tetrazolium (NBT)
1.9. 5-bromo 4-chloro 3-indolyl phosphate (BCIP)
1.10. Oligomix 5 probe
1.11. Hybridisation oven (Hybaid Midi Dual 14) or water bath
1.12. Piece of support mesh (for use with hybridisation oven)
1.13. Hybridisation bottle (for use with hybridisation oven)
1.14. Hybridisation bag (for use with water bath)
1.15. Conical flask or suitable container
1.16. Measuring cylinder
1.17. Heated magnetic stirrers/fleas
1.18. Plastic sandwich boxes that are bigger than the membrane
1.19. Heated bag sealer

METHOD

2.0 Setting up the hybridisation oven [NOTE: use water bath if a hybridisation oven is not available]

2.1. The Hybaid hybridising ovens are fitted with a digital temperature controller. This includes an LED contained in the panel above the four control buttons marked: SET, ▲,▼, and ENT. The temperature of the oven is normally displayed.

2.2. Switch the oven on by pressing the on/off switch. To set the oven to the temperature required, press the SET key once to display SP (set point) and the set temperature flashes alternately.

2.3. Use the ▲ and ▼ keys as necessary to alter the temperature to the required operating temperature (e.g., if the membranes are prehybridised at 68°C, the required operating temperature would be 68°C).

2.4. Finally, press the SET key to commence operation. To view the set point at any time, press the SET key once and then press it again to commence operation. When the required temperature has been set leave the oven for approximately 20 minutes to one hour to stabilise at the set temperature before use. During this initial stabilisation period, the temperature in the oven may rise above the temperature that has been set. This is normal.

3.0 Prehybridisation

3.1. Pre set the oven or water bath to 41°C. If using the oven select a piece of support mesh appropriate for the size of the membrane [follow steps 3.2 to 3.6]. If using a water bath, prepare a hybridisation bag [follow steps 3.7 to 3.8].

3.2. Wet the mesh and hybridisation membrane in a suitable tray containing 10ml of DIG easy hybridisation solution.

3.3. Ensuring that the hybridisation membrane exactly overlays the mesh, roll both up into a tight roll. Transfer the roll to a hybridisation bottle and add 20ml of hybridisation solution.

3.4. Place the bottle on a flat surface and then slowly unwind the membrane and mesh around the inside of the bottle by rocking and gently rolling the bottle along the surface. No air bubbles should be visible between the membrane and the bottle. If bubbles are present the membrane should be removed and rerolled. The procedure should be repeated more gently. Rock the bottle backwards and forwards to attach the first part of the membrane to the bottle. Then roll the bottle in order to unwind the mesh and membrane.

3.5. Once the membrane is in place, replace the cap on the bottle and clip into place on the rotisserie. The rotisserie should be balanced in the same manner as a centrifuge. If there are insufficient bottles in the oven, then load empty bottles in order to create the balance.

3.6. Ensure that the bottles are placed in the rotisserie such that the bottles will rotate in the rotisserie in the same direction as the membranes are rolled. If this is not done, the membranes may roll itself back during pre-hybridisation. If this does occur, simply remove the bottle from the rotisserie and replace in the reverse orientation with the cap on the other side of the rotisserie. The membrane will then unwind itself during the rotation.

3.7. Place the dry membrane in a hybridisation bag, add 20ml of DIG easy hybridisation solution, and expel most of the air bubbles before heat-sealing the bag. Use a fine-tipped pipette to suck the air bubbles out, if useful.

3.8. Ensure the bag is water-tight before placing in the water bath. Place weights on the corners of the bag, making sure not to put weight onto the membrane, to secure the bag at the bottom of the water bath.

3.9. The membrane should prehybridised for 30-60mins.

4.0 Hybridisation [follow steps 4.1 to 4.3 for hybridisation oven or 4.4 to 4.7 for water bath]

4.1. Remove the hybridisation bottle from the oven.

4.2. Unscrew the cap and pour off the solution in it.

4.3. Dispense 3.5ml of DIG easy hybridisation solution and 2pmol of Oligomix 5 probe/ml of hybridisation solution into the hybridisation bottle. Replace the cap and gently agitate the bottle to ensure an even distribution of the probe in the buffer. Incubate for 4 hours at 41°C.

4.4. Remove the hybridisation bag from the water bath.

4.5. Snip off the corner of the bag and remove the DIG easy hybridisation solution.

4.6. Replace with 3.5ml of DIG easy hybridisation solution and 2pmol of Oligomix 5 probe/ml of hybridisation solution into the hybridisation bag. Expel most of the air bubbles before resealing the bag.

4.7. Ensure the bag is water-tight before placing in the water bath. Place weights on the corners of the bag, making sure not to put weight onto the membrane, to secure the bag at the bottom of the water bath. Incubate for 4 hours at 41°C.

5.0 Washing

5.1. After the membrane has been hybridised at 41°C, remove the bottle from the oven. Pour off the solution inside the bottle. Set the temperature of a water bath to 41°C for the washes.

5.2. Wearing gloves, carefully take the mesh and the membrane out of the bottle.

5.3. Unroll the piece of mesh, lift the membrane out carefully and place in a clean plastic sandwich tray.

5.4. Pour 100ml of Washing Solution A into the tray and wash the membrane in the buffer by placing the tray on the shaker for five minutes in the water bath.

5.5. After five minutes, pour off the solution and wash the membrane as follows at 41^{o}C: 2 x 5 minutes in 200ml Washing Solution A and 2 x 5 minutes in 200ml Washing Solution B.

6.0 Detection of DIG

6.1. Transfer membrane to a clean plastic box and incubate the membrane for 30minutes in 100ml Buffer 2 on a shaker.

6.2. Dilute the alkaline phosphatase conjugated anti-DIG antibody 1:4000 in Buffer 1. For each hybridisation tube dilute 20µl antibody in 80ml of Buffer 1. Incubate the membrane in the appropriate conjugate solution at room temperature for 30 minutes on a shaker.

6.3. After incubation, pour off the conjugate solution. Remove unbound conjugate by washing the membrane in 200ml of Buffer 1 for 15 minutes. Equilibrate the membrane in 100ml of Buffer 3 in the plastic box for 5 minutes on a shaker.

6.4. Take the membrane out of the box and place it in a hybridisation bag or a plastic bag. Add 50ml of Buffer 3 supplemented with 225µl of NBT (75mg/ml) and 175µl of BCIP (50mg/ml). Exclude air from the bag and seal the bag with a bag sealer.

6.5. Spread the contents by gently stroking the bag.

6.6. Cover with foil and place the membrane flat in a dark place at room temperature and let it develop without shaking.

6.7. The bands should start to appear after approximately two hours. However the membranes should be incubated for approximately 16 hours.

Note: If it takes 24 hours or more for all the bands to come up, the membrane should be left to develop at 4°C for the remaining time. If the membrane is not placed at 4°C, the background will come up faster than the bands and the membrane will go black.

6.8. As soon as the bands are visible, remove the filter from the bag and place it in a clean plastic sandwich tray and wash the filter with 200ml of sterile distilled water.

6.9. Dry the membrane between two sheets of filter paper and dry at 37°C. Store in a plastic bag.

The results can be documented and analysed by scanning. The image can then be analysed by Taxotron®, BioNumerics, or a similar software package.

A. Preparation of Solutions required for Extraction and Restriction Endonuclease Digestion of *Corynebacterium diphtheriae* and *Citrobacter koseri*

SUMMARY
This section describes the methods for the preparation of solutions required for the extraction of and restriction endonuclease digestion of *C. diphtheriae* and *Citrobacter koseri* DNA. For *C. diphtheriae,* GES Reagent is used to lyse the bacterial cells, ammonium acetate and chloroform/isoamylalchol to precipitate out the proteins and isopropanol to precipitate out the DNA. For *Citrobacter koseri,* lysis buffer is used to lyse the bacterial cells, phenol/chloroform precipitates the proteins and ethanol precipitates the DNA. The solutions described can be made up and stored until required.

SAFETY
This procedure must not be performed by pregnant workers. Gloves and safety glasses must be worn at all times. Phenol is highly corrosive and must be handled in a fume cupboard. Phenol burns, in the event of contact with skin, eyes, etc wash with water, cover with glycerol and seek medical advice immediately. Chloroform must not be handled by pregnant workers.

1.0 MATERIALS

1.1. Guanidium thiocyanate
1.2. 30% Sarcosyl
1.3. Ammonium acetate
1.4. EDTA
1.5. NaOH
1.6. Phenol
1.7. Chloroform
1.8. Isoamylalcohol
1.9. Tris Borate EDTA (TBE) sachets
1.10. Tris hydrochloride
1.11. Concentrated hydrochloric acid
1.12. Ficoll
1.13. Bromophenol Blue
1.14. Sterile distilled water
1.15. Waterbath
1.16. 50ml measuring cylinders
1.17. 100ml measuring cylinders
1.18. 250ml beakers
1.19. 100ml Schott bottles or suitable containers
1.20. Heated magnetic stirrers/fleas
1.21. Spatulas
1.22. Weighing boats or suitable containers
1.23. Disposable Pasteur pipettes
1.24. Universal or suitable containers

1.25. 5L flask or suitable container
1.26. Absolute Ethanol

METHOD

2.0 Preparation of 100mL of 5M NaOH

NaOH is corrosive. This chemical must be handled in a fume cupboard.

2.1. Weigh out 20g of NaOH and put it into a 250ml beaker. Using a measuring cylinder, add 80ml of sterile distilled water to the NaOH in the beaker.

Care must be taken because the beaker will become hot because, when NaOH reacts with water, heat is released.

2.2. Dissolve the NaOH by placing the beaker on a magnetic stirrer.

2.3. Make up the solution to 100ml using a 100ml measuring cylinder. Transfer solution to a schott bottle or suitable container. Store at room temperature in a suitable cupboard. Shelf life is up to two years.

3.0 Preparation of 100mL of EDTA pH7.0

3.1. Weigh out 93.05g of EDTA and put it into a 250ml beaker.

3.2. Add 50ml of sterile distilled water using a measuring cylinder to the EDTA.

3.3. Dissolve the EDTA by placing the beaker on a heated magnetic stirrer.

3.4. Adjust the pH of the solution to 7.0 by adding 5M NaOH (3.0), one drop at a time.

3.5. Transfer solution to a schott bottle or suitable container. Store at room temperature in a suitable cupboard. Shelf life is up to two years.

4.0 Preparation of 1M TRIS-HCL solution

These reagents must be handled in the fume cupboard.

4.1. Weigh out 12.1g of Tris hydrochloride and put it into a 250ml beaker.

4.2. Add 50ml of sterile distilled water to the beaker containing the Tris and dissolve it by placing the beaker on a magnetic stirrer.

4.3. Once dissolved, adjust the pH of the solution to pH7.0 by using concentrated hydrochloric acid (Conc HCl) a drop at a time using a 1ml plastic disposable pasteur pipette.

4.4. Make up the solution to 100ml by using a measuring cylinder. Transfer solution to a schott bottle or suitable container. Store at room temperature in a suitable cupboard.

Alternatively, Tris HCl solution can be bought from Sigma.

5.0 Preparation of TE buffer pH7.0

5.1. Add 1ml of 1M Tris-HCl solution and 20µl of 0.5M EDTA into a 100ml measuring cylinder.

5.2. Make up to 100ml with sterile distilled water. Transfer solution to a schott bottle or suitable container. Store at room temperature in a suitable cupboard.

6.0 Preparation of LYSIS buffer

6.1. Add 29ml of TE Buffer into a 250ml beaker.

6.2. Add 1ml of 30% sarkosyl. Mix well and transfer solution to a schott bottle or suitable container. Store at room temperature in a suitable cupboard.

7.0 Preparation of GES reagent – FOR *C. DIPHTHERIAE* ONLY

These reagents must be handled in the fume cupboard. GES reagent contains guanidine thiocyanate so HEATING TO DECOMPOSITION OR CONTACT WITH ACIDS OR ACID VAPORS CAN LIBERATE POISONOUS CYANIDE VAPOURS. As GES reagent is a toxic substance, storage containers must display a poisons label.

7.1. Weigh out 59.08g of guanidium thiocyanate and put it into a 250ml beaker.

7.2. Add 20ml of 0.5M EDTA pH 7.0 into the beaker and dissolve by placing it in a 50°C water bath.

7.3. Once the guanidium thiocyanate has dissolved, add 1.7ml of 30% sarcosyl and then make up the solution to 100ml by using a measuring cylinder.

7.4. Transfer solution to a schott bottle or suitable container. As GES reagent is a toxic substance, storage containers must display a poisons label. Store at room temperature in a suitable locked cupboard.

8.0 Preparation of 100ML of 7.5M Ammonium Acetate – FOR *C. DIPHTHERIAE* ONLY

This chemical must be handled in the fume cupboard.

8.1. Weigh out 57.81g of ammonium acetate and put it into a 250ml beaker.

8.2. Measure 50ml of distilled water using a measuring cylinder and add it to the ammonium acetate.

8.3. Dissolve the ammonium acetate by placing the beaker on a magnetic stirrer.

8.4. Once the ammonium acetate has dissolved make up the solution to 100ml using a measuring cylinder.

8.5. Transfer solution to a schott bottle or suitable container. Store at room temperature in a suitable cupboard.

9.0 Preparation of PHENOL/CHLOROFORM – FOR *CITROBACTER KOSERI* ONLY

Prepare phenol/chloroform in the fume cupboard. Nitrile gloves and safety glasses should be worn. Phenol is highly corrosive. Phenol burns, in the event of contact with skin, eyes, etc wash with water, cover with glycerol and seek medical advice immediately. Chloroform must not be handled by pregnant workers.

9.1. To make 1 litre of phenol/chloroform, add 500ml of phenol and 500ml of chloroform into a measuring cylinder.

9.2. Transfer solution to a schott bottle or suitable container. As phenol/ chloroform is a toxic substance, the storage container must display a poisons label.

9.3. Store at room temperature in a locked cupboard.

10.0 Preparation of 100ML of Chloroform/Isoamylalcohol (24:1) – FOR *C. DIPHTHERIAE* ONLY

These reagents must be handled in the fume cupboard. As chloroform is a toxic substance, storage containers for this reagent must display a poisons label. Chloroform should not be handled by pregnant workers.

10.1. Chloroform and isoamylalcohol are mixed in the proportions 24:1 in a 250ml beaker.

10.2. To prepare 100ml of the above solution, add 96ml of chloroform to 4ml of isoamylalcohol.

10.3. Transfer solution to a schott bottle or suitable container. As chloroform is a toxic substance, the storage container must display a poisons label.

10.4. Store at room temperature in a locked cupboard for flammable liquids.

11.0 Preparation of 5 litres of 1X Tris Borate EDTA (TBE)

11.1. Empty the contents of the TBE sachet into a 5 litre flask or a suitable container. Add 4 litres of distilled water. Dissolve the TBE by placing the container on a magnetic stirrer.

11.2. Make up the volume to 5 litres. Transfer solution to a suitable container.

11.3. Store at room temperature in a suitable cupboard.

12.0 Preparation of 10ML of 10% Ficoll Bromophenol Blue (BPB)

12.1. Weigh out 2g of Ficoll into a universal or a suitable container.

12.2. Add 10ml of 1xTBE into the container. Dissolve the Ficoll by placing the container on a heated magnetic stirrer.

12.3. Once the Ficoll has dissolved, make up the solution to 20ml and add a very small amount of BPB powder (final concentration 0.05%) to the Ficoll until the solution becomes a deep purple/blue colour. Store at room temperature in a suitable cupboard.

13.0 Preparation of 100ML of 70% Ethanol

13.1. Wear gloves and safety glasses. Ethanol is flammable and this procedure should be carried out in a fume cupboard away from naked flames

13.2. In to a 100ml Schott bottle measure out 70ml absolute ethanol. Add 30ml sterile distilled water and mix. Store the bottle in a flammable cupboard until use.

B. Preparation of Solutions required for the Agarose Gel Electrophoresis and Southern Blotting of *Corynebacterium diphtheriae*

SUMMARY
This section describes the method for the preparation of solutions required for the agarose gel electrophoresis and Southern Blotting of *C. diphtheriae* DNA restriction fragments. The solutions described can be made up and stored to be used for future use.

SAFETY
This procedure must not be performed by pregnant workers.
Nitrile gloves must be worn at all times.

1.0 MATERIALS

1.1. Sodium chloride (NaCl)
1.2. Sodium hydroxide (NaOH)
1.3. 20x SSC
1.4. Ficoll
1.5. Bromophenol Blue
1.6. Sterile distilled water
1.7. Measuring cylinders
1.8. Schott bottles or suitable containers
1.9. Heated magnetic stirrers/fleas
1.10. Spatulas
1.11. Weighing boats or suitable containers
1.12. Disposable Pasteur pipettes
1.13. Universal or suitable container
1.14. 1 litre beaker or a suitable container
1.15. 5 litre flask or suitable container

METHOD

2 Preparation of 300ML of Denaturing Solution (PREPARE ON THE DAY OF USE)

Caution: NaOH is corrosive. This chemical must be handled in the fume cupboard. Nitrile gloves and safety glasses must be worn at all times.

2.1 Weigh out 26.28g of NaCl and 6g of NaOH in to a 1 litre beaker or suitable container.

2.2 Measure 250ml of distilled water using a 1 litre measuring cylinder and add the water slowly to the NaCl and the NaOH.

Care must be taken because the container will become hot because, when NaOH reacts with water, heat is released.

2.3 Dissolve the contents by placing the container on a magnetic stirrer.

2.4 Once dissolved, make up the volume to 300ml using a measuring cylinder with sterile distilled water. Let the solution stir for a little longer to make sure that the solution is thoroughly mixed. Store the solution in a suitable container at room temperature.

3 Preparation of 200ML of Transfer Solution.

Caution: NaOH is corrosive. This chemical must be handled in the fume cupboard. Nitrile gloves and safety glasses must be worn at all times.

3.1 Weigh out 17.56g of NaCl and 2g of NaOH in a 1 litre beaker or a suitable container.

3.2 Measure 100ml of distilled water and add the water slowly to the NaCl and the NaOH.

Care must be taken because the container will become hot because, when NaOH reacts with water, heat is released.

3.3 Dissolve the contents by placing the container on a magnetic stirrer.

3.4 Once dissolved, make the volume to 200ml using a measuring cylinder with sterile distilled water. Let the solution stir for a little longer to make sure that the solution has thoroughly mixed. Store the solution in a suitable container at room temperature.

4 Preparation of 1 litre of 2x SSC

4.1 20x SSC can be sourced from Sigma. 2x SSC is made by diluting 20x SSC.

4.2 Pour 100ml of 20x SSC in to a 1 litre flask or a suitable container. Dilute the 20x SSC to 2x SSC by adding 900ml of sterile distilled water to the 100ml of 20x SSC. Mix well and store in a suitable container at room temperature

C. Preparation of Solutions required for the Prehybridisation, Hybridisation and Detection of Hybrids used in the Ribotyping of *Corynebacterium diphtheriae*

SUMMARY
This section describes the preparation of solutions required for the prehybridisation, hybridisation and immunological detection of hybrids in the ribotyping procedure for *C. diphtheriae*.

SAFETY
Nitrile gloves and safety glasses must be worn at all times. Sodium dodecyl sulphate (SDS) and concentrated hydrochloric acid (HCl) must be handled in a fume cupboard. SDS is irritating to the eyes and skin.

1.0 MATERIALS

1.1. 20x SSC
1.2. 30% sarkosyl
1.3. Sodium dodecyl sulphate (SDS)
1.4. Tris hydrochloride (Tris-HCl)
1.5. Sodium chloride ($NaCl_2$)
1.6. Magnesium chloride ($MgCl_2$)
1.7. Concentrated hydrochloric acid (conc HCl)
1.8. Beakers
1.9. 1ml pipette and tips
1.10. Heated magnetic stirrer
1.11. Measuring cylinders
1.12. Schott bottles or suitable containers
1.13. Weighing boats or suitable containers
1.14. 500ml beakers or a suitable containers
1.15. DIG blocking reagent (Roche Diagnostics 1096176)

METHOD

2.0 Preparation of 500ml of 10% SDS

This chemical must be handled in the fume cupboard.

2.1. Weigh out 50g of SDS in a weighing boat or suitable container and put into a 500ml beaker or suitable container.

2.2. Add 250ml of sterile distilled water using a measuring cylinder.

2.3. Dissolve the SDS by placing the beaker on a magnetic stirrer.

2.4. Once the SDS has dissolved, make up the solution to 500ml using a suitable measuring cylinder. Transfer solution to a suitable container and store the solution at room temperature in a suitable cupboard.

3.0 Preparation of 500ML of 1M TRIS-HCl PH7.5

These chemicals must be handled in the fume cupboard. Nitrile gloves and safety glasses must be worn at all times.

3.1. Weigh out 60.57g of Tris-HCl in a 500ml beaker or suitable container.

3.2. Add about 250ml of sterile distilled water using a measuring cylinder.

3.3. Dissolve the Tris-HCl by placing the container on a magnetic stirrer.

3.4. When all the Tris-HCl has dissolved, the solution should be pH tested and adjusted to pH7.5 using concentrated HCl.

3.5. Using a disposable Pasteur pipette, drop by drop add concentrated HCl to the Tris-HCl solution in the beaker, while it is stirring slowly on a magnetic stirrer. The pH should be checked using pH paper after the addition of every few drops, till the pH decreases to 7.5.

3.6. Make up the solution to 500ml with distilled water using a suitable measuring cylinder.

3.7. Pour the solution into a suitable container for storage and store at room temperature in a safe place.

Alternatively, Tris HCl solution can be bought from Sigma.

4.0 Preparation of 500ML of 1M TRIS-HCl PH9.5

These chemicals must be handled in the fume cupboard. Nitrile gloves and safety glasses must be worn at all times.

4.1. This solution is prepared in the same way as Tris-HCl pH7.5. The only difference is that the pH is 9.5, which means that less concentrated HCl needs to be added to the solution.

Alternatively, Tris HCl solution can be bought from Sigma.

5.0 Preparation of 500ML of 1M Magnesium Chloride ($MgCl_2$)

5.1. Weigh out 101.7g of $MgCl_2$ into a weighing boat or suitable container.

5.2. Put the $MgCl_2$ into a 500ml beaker or suitable container and add 250ml of sterile distilled water.

5.3. Dissolve the $MgCl_2$ by placing the container on a magnetic stirrer.

5.4. Once all the $MgCl_2$ has dissolved, retrieve the flea and make up the volume to 500ml with sterile distilled water using a suitable measuring cylinder.

5.5. Pour the solution into a suitable container for storage and store at room temperature in a safe place.

6.0 Preparation of 500ML of 5M Sodium Chloride ($NaCl_2$)

6.1. Weigh out 146.1g of $NaCl_2$ into a weighing boat or suitable container.

6.2. Put the $NaCl_2$ into a 500ml beaker or suitable container and add 250ml of sterile distilled water.

6.3. Dissolve the $NaCl_2$ by placing the container on a magnetic stirrer.

6.4. Once all the $NaCl_2$ has dissolved, retrieve the flea and make up the volume to 500ml with sterile distilled water using a suitable measuring cylinder.

6.5. Pour the solution into a suitable container for storage and store at room temperature in a safe place.

Alternatively, NaCl solution can be bought from Sigma.

7.0 Preparation of Wash Solution A (2 X SSC, 0.1% (W/V) SDS; 400ML)

Note: Always prepare this buffer on the day of the prehybridisation, hybridisation and detection of hybrids in the ribotyping procedure

7.1. Take a 1 litre measuring cylinder and add 40ml of 20x SSC solution.

7.2. Add 356ml of sterile distilled water into the measuring cylinder containing the 40ml of 20x SSC.

7.3. Add 10ml of 10% SDS solution and pour the solution into a suitable container for storage.

7.4. Place in a waterbath set at 37°C to dissolve as the solution may become slightly cloudy. Store until ready to use at room temperature.

8.0 Preparation of Wash Solution B (0.1 X SSC, 0.1% (W/V) SDS; 400ML

Note: Always prepare this buffer on the day of use

8.1. Take a 1 litre measuring cylinder and add 2ml of 20x SSC solution.

8.2. Add 394ml of sterile distilled water into the measuring cylinder containing the 2.5ml of 20x SSC.

8.3. Add 4ml of 10% SDS solution and pour the solution into a suitable container for storage. Mix the solution and store until ready to use at room temperature. Equilibrate to 68°C before use.

9.0 Preparation of 1L of Buffer 1 (0.1M TRIS HCl, 0.15M NaCl, PH7.5)

9.1. Take a 1 litre measuring cylinder and put 100ml of 1M Tris-HCl pH7.5 and 30ml of 5M NaCl into it.

9.2. Add 870ml of sterile distilled water into the measuring cylinder.

9.3. Cover the mouth of the measuring cylinder with some cling film and mix the solution by carefully inverting the measuring cylinder up and down.

9.4. Pour the solution into a suitable container for storage and store at room temperature in a safe place.

10.0 Preparation of 100ML of Buffer 2 (1% (W/V) REACTION BLOCKER, 0.1M TRIS-HCl, 0.15M NaCl PH 7.5).

PREPARE ON THE DAY, APPROXIMATELY 1 HOUR BEFORE USE.

10.1. Weigh out 1g of DIG blocking reagent into a weighing boat or a suitable container, and put it into a 250ml flask or a suitable container.

10.2. Add 90ml Buffer1 into the container. Dissolve the blocking reagent by placing the flask on a heated magnetic stirrer. Take care; the heated stirrer can become very hot.

10.3. When the entire blocking reagent has dissolved, wear gloves to remove the hot flask from the stirrer. **The flask can get very hot and care should be taken when removing the flask.** Make the volume up to 100ml with Buffer 1 using a suitable measuring cylinder.

10.4. Pour into a suitable container and, if this solution is made prior to the day of use, it can be stored at -20°C. When the buffer is required for use, it needs to be thawed by placing the container in a warm waterbath or by simply leaving it on the bench at room temperature. If the buffer is hot it should be cooled to room temperature before use.

11.0 Preparation of 200ML of Buffer 3 (0.1M TRIS HCl, 0.1M NaCl, 50mM $MgCl_2$ PH 9.5)

11.1. Take a 1 litre measuring cylinder and put 20ml of 1M Tris-HCl ph9.5, 4ml of 5M NaCl and 10ml of 1M $MgCl_2$ into it using suitable measuring cylinders.

11.2. Add 166ml of sterile distilled water into the measuring cylinder.

11.3. Cover the mouth of the measuring cylinder with some cling film and mix the solution by carefully inverting the measuring cylinder up and down.

Pour the solution into a suitable container for storage and store at room temperature in a safe place.