PROTOCOL 5. DETECTION OF VIRULENCE GENES BY HYBRIDIZATION WITH DNA PRODES

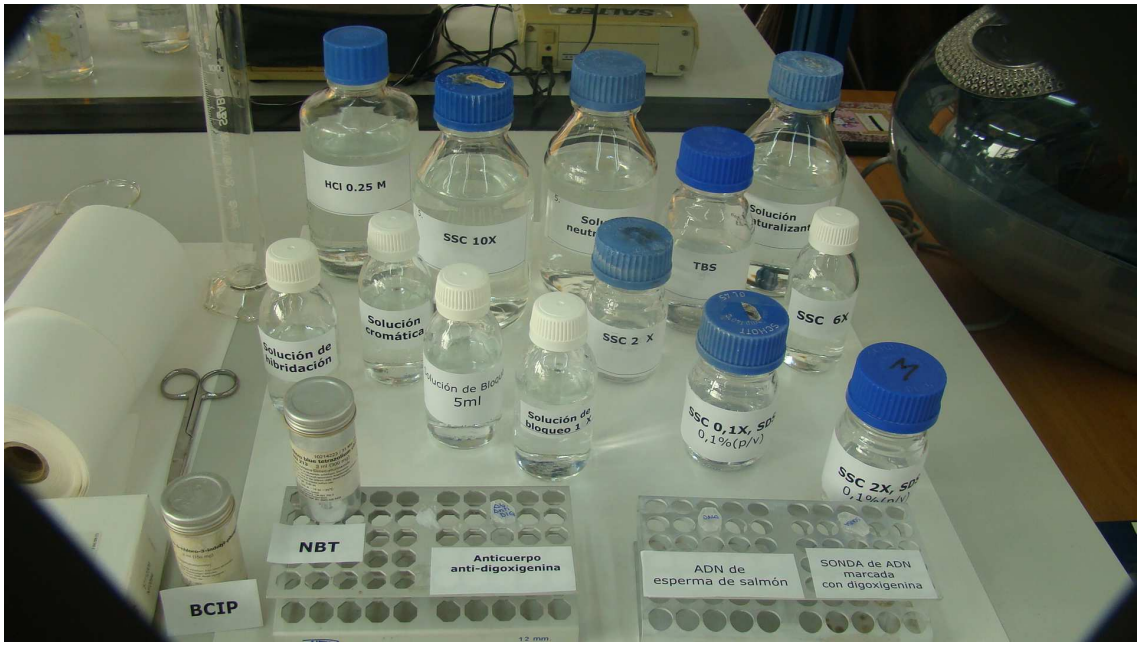
Materials

- Eppendorf tubes (vol 1,5 ml), sterile
- Eppendorf tubes thin-wall (vol 0.5 ml) for PCR reactions de
- Pipettes and tips, sterile
- Gloves
- Biological cabinet
- Termocycler
- Transilluminator
- Paper tissues
- Nylon membranes
- Equipment for capillary transfer
- Whattman 3MM
- Aluminium foil
- Agarose
- Electrophoresis chamber
- Cutter
- Hybridization oven
- Parafilm
- Hybridization bags
- Hand-held electric sealer
- Laboratory platform shaker

Solutions

- Molecular grade distilled water, sterile
- *Taq* polimerase buffer 10 X
- 50 mM Magnesium chloride
- Primers
- dATP, dGTP, y dCTP: 0.2 mmol/l each
- DIG-11-dUTP: 0.07 mmol/l
- dTTP: 0.13 mmol/l
- *Taq* polymerase 5 U/ µl
- Molecular weight marker, digoxigenin-labeled

- 1 X TBE
- 3M Sodium acetate (pH 5.2): Dissolve 40.8 g sodium acetate in distilled water. Adjust to pH 5.2 with acetic acid, then to 100ml volume with distilled water and autoclave.
- Chilled etanol
- TE (pH 8.0): 10 mM Tris-HCl**,** 1mM EDTA. Autoclave and keep at room temperature
- Salmon sperm DNA solution
- SSC 20X: Dissolve 175.3 g of NaCl and 88.2 g sodium citrate in distilled water. Adjust to 1 L and autoclave.
- 0.25 M HCl: Mix 21.5 ml 11.6 M HCl (d. 1.18) and 978.5 ml distilled water. Keep at room temparature.
- Denaturation solution: 1.5 M NaCl, 0.5 M NaOH
- Neutralization solution: 0.5 M Tris-HCl pH 7.5, 1.5 M NaCl
- Deionized formamide: Mix 10 ml formamide with 5-10 g de ionic interchange resine. Mix during 30 minutes, filter with Whatman 3MM. and keep at - 20 °C.
- Hybridation solution: 50 %: 5 X SSC, blocking solution 2% (w/v), N-lauroylsarcosine, sodium salt 0.1 % (w/v) , SDS 0.02 % (w/v); 50 % deionized formamide. When used, add 100 µg of denaturated salmon sperm DNA (100 °C for 10 minutes).
- Blocking solution 10 X: 10 g blocking reagent, 100 ml maleic aqcid buffer. Dissolve using a microwave oven . Keep at -20 °C.
- Antidigoxigenin antibody
- Maleic acid buffer (pH 7.5): 11.6 g mleic acid, 30 ml 5M NaCl, distilled water to1000 ml.
- TBS: 0.05 M Tris-HCl, 0.15 M NaCl; pH 7.5
- Detection buffer: 0.1 M Tris-HCl (pH 9.5); 0.1 M NaCl. Prepare freshly and protect from direct light before use. To prepare 10 ml mix 50 µl NBT and 37.5 µl BCIP (Roche diagnostics)

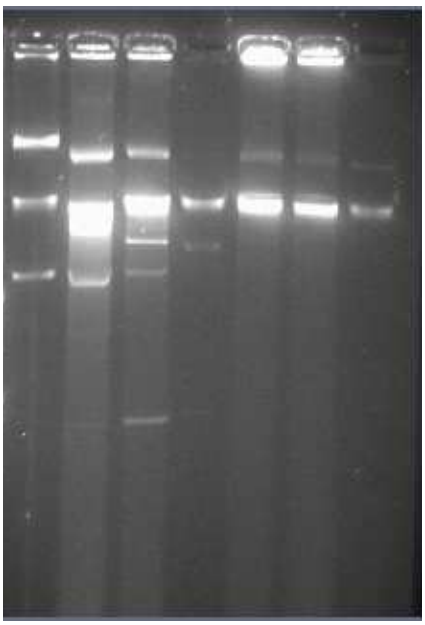


Procedure (adapted from the "The DIG System User´s Guide for Filter Hybridization", Roche Diagnostics)

SOUTHERN BLOTTING

5.A. <u>Southern transfer</u>.

This is a technique for detecting particular DNA fragments, defined in terms of their sequence. DNA molecules are separated by agarose gel electrophoresis and transferred to a solid support. This image of the gel is then probed with a known, labelled DNA, which hybridises with its complementary counterparts. On visualisation of the label, the size of the molecule bearing sequences complementary to that of the probe is revealed.



1. Take a photograph of the gel and place it on a tray.
2. Soak gel in dilute 0.25 M HCl for 15 min
3. Soak for 2 × 15 min, at room temperature, in denaturation solution with gentle shaking.
4. Soak for 2 × 15 min, at room temperature, in neutralization solution with gentle shaking.
5. Transfer to the solid support:

- Wet filter in water, then place in 2X SSC.
- Place a glass plate on suitable supports in a plastic box
- Cut 3MM paper wicks to fit, and place over the glass plate so that the ends dip to the bottom of the box . Use two wicks at right angles to each other. Pour 10 X SSC on the paper to wet it.
- Place the gel on the wicks
- Place the filter on the gel and cut one corner off for registration purposes
- Place strips of parafilm along each side of the gel (the idea is to prevent buffer bypassing the gel)
- Take three pieces of 3MM paper cut to the size of the gel, wet them in 10 X SSC and place on top of the filter.
- Put a pile of tissues or paper towels 2-4 inches high on top of the 3MM paper.
- Put a heavy (500-1000 g) weight on top and make sure it is well balanced.
- Fill the box with 10 X SSC to just below the level of the glass plate and leave overnight.
- Carefully dismantle the pile, extract the filter and rinse gently with 2X SSC
- Dry at 37ºC between sheets of filter paper until completely dry.
- Expose to UV light on a transilluminator, DNA side down for 5 min.

5.B. <u>Obtention of a digoxigenin-labelled DNA probe</u>

1. Prepare a master mix under the same conditions as amplification of the corresponding gene but adding nucleotides as follows:

- dATP, dGTP, and dCTP: 0.2 mmol/l each
- DIG-11-dUTP: 0.07 mmol/l
- dTTP: 0.13 mmol/l
- molecular biology grade water

2. Precipitate DNA adding 0.1 volumes of 3M sodium acetate and 2 volumes of chilled ethanol. Keep at - 20 °C, overnight. Centrifuge at 12000 rpm for 20 min and resuspend in TE.

Estimate the yield of Dig-labelled probe: dilution series of the labeling reaction and dilutions of an appropriate standard are both spotted on nylon membranes. The membrane is then processed in a short detection procedure.

5.C. Hybridization

The procedure is divided into three steps : prehybridization, hybridization and posthybridization washes.

1. Prehybridization. Place the blot in a hybridization bag containing 20 ml prehybridization solution per 100 cm^2 of membrane surface area. Seal the bag, and prehybridize at 42 °C for 1-2 hours. The membrane must be covered by sufficient solution to move freely in the bag.

2. Hybridization. Discard the prehybridization solution from the bag. Add the hybridization solution (2.5 ml prehybridization solution per 100 cm^2 of membrane surface area) containing the Dig-labeled probe (10-15 ng per ml) and incubate the membrane overnight at 42 °C. Whenusing double-stranded DNA probes, heat in a boiling water bath for ten min to denature the DNA. Chill directly on ice.

3. Posthybridization washes. At the end of the hybridization, pour the solution from the bag. Wash the membrane twice, at room temperature, in 2 X SSC, SDS 0.1 % (w/v) for 15 minutes and, twice at 68 °C in 0.1 X SSC, SDS 0.1 % (w/v) for 30 minutes.

5.D. Detection of hybrids

1. Incubate membrane for 30 min in 20 ml of blocking buffer 1 X with gently agitation.

2. Wash the membane 3x 10 minutes in 50 ml of TBS.

3. Dilute the antibody 1:5000 in blocking solution (20 ml per 100 $cm^{2)}$ and incubate the membrane for 1 hour

4. Wash the membane 3x 10 minutes in 50 ml of TBS.

5.Equilibrate membrane in 20 ml detection buffer.

6. Pour off the detection buffer and add approximately 10ml color substrate solution to the membrane. Incubate the membrane in a sealed plastic bag or box in the dark. Do not shake the container while the color is developing. The color precipitate stars to form within a few min, and the reaction is usually completed after 12 hours.

7. Once the desired spots or bands are detected, wash the membrane with water to prevent over-development.

Results can be documented by photocopying the wet filter or by photography.