1. Slot blot

1.1. Isolation of genomic DNA

All genomic DNA that were isolated in the present thesis was as per the modified protocol of Doyle and Doyle 1990.

- Sample (0.5 g) was ground to fine powder after freezing the tissue in liquid nitrogen with the help of a pestle and mortar.
- The powder was transferred to a centrifuge tube containing 4 ml of pre-warmed (65°C) DNA extraction buffer and mixed vigorously by vortexing.
- Incubated the above at 65°C for 1 h with occasional mixing.
- Added equal volume of chloroform: iso-amyl alcohol (24:1) and mixed gently by inverting the tube for 5 min.
- Centrifuged the mixture at 15,000 rpm at 25°C for 20 min.
- The upper layer (aqueous phase) was transferred to fresh centrifuge tube with a wide bore pipette and added 0.6 volume of iso-propanol and mixed by gentle inversions.
- DNA was precipitated by centrifugation at 15,000 rpm at 4°C for 30 min and the pellet obtained was washed with 5 ml of chilled 70% ethanol.
- After draining off the ethanol the pellet was air-dried and finally dissolved in a minimum volume of TE buffer.
- Transferred the above to 1.5 ml eppendorf and added 10 μg/ml of the RNase A stock to this, mixed well and incubated at 37°C for 1 h.

<table>
<thead>
<tr>
<th>TE Buffer</th>
<th>RNase A (20 mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris (pH 8; 10mM)</td>
<td>Dissolve RNase A in 10 mM Tris-HCl (pH 7.5) and 15 mM NaCl, then heat at 100°C for 15 min followed by cooling at room temperature. Store as aliquots at -20°C</td>
</tr>
</tbody>
</table>
Added equal volumes of phenol: chloroform: iso-amyl alcohol (25:24:1) and mixed gently by inverting the tube followed by centrifugation at 13,000 rpm for 20 min at 25°C.

Upper aqueous phase was collected and subjected to chloroform: iso-amyl alcohol extraction.

Transferred the upper aqueous phase to a fresh tube and precipitated the DNA by centrifugation at 15,000 rpm for 30 min at 4°C after the addition of 0.1 volume of 3M sodium acetate and double the volume of chilled ethanol.

**3M Sodium acetate**
Dissolve 24.609 g sodium acetate in 100 ml of autoclaved distilled water and adjust the pH to 4.8 with glacial acetic acid.

The pellet was washed once with 70% ethanol to remove the extra salts and after air drying, finally dissolved the DNA in a minimum volume of TE buffer and checked on 0.8% agarose gel.

The DNA was stored at -20°C after quantification.

**DNA extraction buffer**

<table>
<thead>
<tr>
<th>Working conc.</th>
<th>Stock conc.</th>
<th>Volume required (for 100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (pH 8)</td>
<td>100 mM</td>
<td>1.0 M</td>
</tr>
<tr>
<td>Na-EDTA (pH 8)</td>
<td>20 mM</td>
<td>0.5 M</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.4 M</td>
<td>4.0 M</td>
</tr>
<tr>
<td>CTAB</td>
<td>2%</td>
<td>10%</td>
</tr>
<tr>
<td>ADW</td>
<td></td>
<td>For making final volume</td>
</tr>
</tbody>
</table>

β-mercaptoethanol 0.2% 0.2 ml

**β-mercaptoethanol was added just prior to use**

1.2. Blotting the membrane

Nylon membrane (Amersham Hybond™-XL, GE Healthcare, Sweden) was cut to appropriate size of dot/slot blot manifold. Different concentrations of the DNA sample (say 15, 30 and 45 ng) were taken and diluted, so as to make a total volume of 6 µl each.
The membrane was placed in 18.2 milliQ water for 5 min with gentle shaking.

MilliQ water was then replaced with 6X SSC followed by 10X SSC buffer and kept for 5 min each with gentle shaking.

Blotting assembly (Hoefer PR 648 Slot blot manifold, Amersham Pharmacia Biotech, USA) was set and the screws were tightened after the nylon membrane was placed on manifold.

Added 100 µl of 10X SSC buffer in each well.

Vacuum dried at 40-60 mbar, till the nylon membrane dried (about 5 min).

DNA was denatured by boiling at 100°C for 10 min, snap cooled on ice and centrifuged briefly.

Denatured DNA samples were loaded on membrane (6 µl) with the help of micropipette and vacuum dried for 2-3 min.

Added 100 µl 10X SSC buffer in each well and vacuum dried for 5-10 min.

Blot the membrane on Whatman filter paper no. 1.

The membrane was then cross-linked twice for 2 min each with the help of UV cross-linker (Hoefer UVC 500, Amersham Biosciences, Inc. USA).

Membrane was wrapped in a cling film and kept at 4°C till further use or until proceed for hybridization.

20X SSC buffer (Sodium chloride and Sodium citrate)

✓ 3 M NaCl (Sigma, USA) - 175.3 g/l (Mw 58.44)
✓ 0.3 M Na-citrate.2H₂O - 88.23 g/l (Mw 294.1)

Dissolved in 800 ml of milliQ H₂O and final volume made to 1 litre with milliQ H₂O after adjusting the pH to 7 with few drops of 14 N HCl. Cover with Al foil and autoclave it.

Diluted 20X SSC buffer with autoclaved milliQ H₂O as per the requirement (10X, 6X, 0.5X, 0.1X SSC).

Nylon membrane was transferred to the hybridization bottle and added 15 ml of pre-hybridization solution (described below; Section 1.7) into the bottle.

Membrane was pre-hybridized at 42°C for 2-3 h at 15-30 rpm in hybridization oven.
Pre-hybridization solution was then replaced with the hybridization solution containing the labelled probe (described below; Section 1.3-1.6). After denaturing the labelled probe (at 100°C for 10 min, immediately kept on ice and spin), added immediately to the 10 ml of fresh pre-hybridization solution. Membrane was hybridized at 42°C for 16 h at 2-5 rpm.

Hybridization solution known as “probe” was removed and can be stored at -20°C for 1-2 uses.

After hybridization the membrane was subjected to stringency washes at 42°C for 15 min by washing the blots twice with 30 ml 2X SSC buffer containing 0.1% SDS at 15-30 rpm.

Again the membrane was washed (twice) at room temperature (25°C) for 30 min each with 0.5X SSC buffer containing 0.1% SDS (30 ml) at 15-30 rpm.

The above buffer was discarded and washed again with 0.1X SSC buffer containing 0.1% SDS (30 ml) for 30 min at 65°C and 15-30 rpm.

Third wash buffer was discarded and 20 ml of 2X SSC buffer was added for rinsing.

The membrane was taken out and dried slightly on Whatman no. 1 filter paper.

The membrane was thus, ready to be used directly for immune-detection or can be stored at 4°C.

1.3. Labelling of probe

The following steps were elucidated below:

1. PCR amplification of the desired fragment with gene specific primers.
2. Elution of the amplified fragment.
3. Labelling of the fragment to be used as probe either by non-radioactive or biotin labelling or radio-labelling.

1.4. Preparing the probe

The probe was prepared by PCR-amplification of the coding/gene sequence using the forward and reverse primers as specified in the ‘Material and Methods’.
1.5. Elution of DNA from gel

The MinElute Gel Extraction Kit from Qiagen was used as follows:

- The DNA fragment from the agarose gel was excised with a clean, sharp scalpel or razor blade. The excess gel was trimmed away to minimize the amount of agarose and the gel slice was weighed in a tared colourless tube.

- Added 3 gel volumes of the Gel Solubilization Solution i.e. buffer QG to the gel slice (for every 100 mg of agarose gel, add 300 µl of buffer QG. For >2% agarose gels, add 6 volumes of buffer QG. The maximum amount of gel slice per spin column is 400 mg; for gel slices >400 mg use more than one MinElute column).

- The gel mixture was incubated at 50°C for 10 min, or until the gel slice was completely dissolved. Vortexed briefly, for 2-3 min during the incubation to help dissolve the gel.

- After the gel slice was dissolved completely, check that the color of the mixture is yellow (similar to buffer QG with no gel slice) prior to proceeding to the next step. If the color of the mixture is orange or violet, add 10 µl of 3M sodium acetate (pH 5) and mix until the colour turns yellow.

- Added 1 gel volume of 100% iso-propanol to the sample and mixed until homogenous.

- MinElute column was placed into one of the 2 ml collection tubes (provided) in a suitable rack.

- To bind DNA, the sample was applied to the MinElute column, and centrifuged for 1 min. If the volume of the sample is >800 µl, load the sample onto the column in 800 µl portions and centrifuged for 1 min after loading the column each time.

- Flow-through was discarded and MinElute column was placed back in the same collection tube.

- Added 500 µl of buffer QG to the spin column and centrifuged for 1 min.

- Flow-through was discarded and placed the MinElute column back in the same collection tube.

- Added 750 µl of Wash Solution i.e. buffer PE to the MinElute column and centrifuged for 1 min.
Discarded the flow-through and centrifuged the MinElute column for an additional 1 min at 13,000 rpm without any additional wash solution to remove excess ethanol (residual ethanol from buffer PE will not be completely removed unless the flow-through is discarded before the final centrifugation).

The MinElute column was transferred to a fresh clean 1.5 ml microcentrifuge tube and added 10 µl of buffer EB (10 mM Tris-Cl, pH 8.5) or water (pH 7-8.5) to the centre of the membrane, let the column stand for 1 min, and then centrifuged for 1 min (elution efficiency is dependent on pH and maximum elution efficiency is achieved between pH 7 and 8.5).

Purified DNA was analyzed on a gel, added 1 volume of loading dye to 5 volumes of purified DNA. The solution was mixed by pipetting up and down before loading the gel.

1.6. Labelling the probe

1.6.1. Biotin labelling

The checked eluted fragment was quantified using NanoDrop.

Firstly, chill the following on ice prior to labelling:

- DNA template to be labelled (about 500 ng) - 10 µl
- Decanucleotide in 5X Reaction buffer - 10 µl
- Water, nuclease free - to make volume to 44 µl

The above components were mixed in 1.5 ml micro-centrifuge tube, vortexed, spin briefly and incubated in a boiling water bath for 5-10 min followed by snap cooling on ice. Spin down quickly.

Add:

- Biotin labelling mix - 5 µl
- Klenow fragment, exo- (5 U) - 1 µl

Shake the tube and spin down briefly. The tube was incubated for about 20 h at 37°C in a water bath.

Reaction was stopped by the addition of 1 µl of 0.5 M EDTA pH 8.0.

After hybridization and stringency washes, the membrane was briefly blot on Whatman filter paper and then incubated for 5 min at room temperature in 30 ml of **Blocking/Washing buffer** on a gel rocker with moderate shaking.
The membrane was blocked in 30 ml of the **Blocking solution** for 30 min at room temperature with moderate shaking.

Incubated the membrane with moderate shaking in 30 ml of freshly prepared diluted Streptavidin-AP conjugate for 30 min at room temperature.

The membrane was washed at room temperature with moderate shaking as follows:

- Incubated twice with 60 ml of **Blocking/Washing buffer** for 15 min.
- Incubated with 30 ml of **Detection buffer** for 10 min and discard the solution.

The membrane was then incubated in 30 ml of freshly prepared **Substrate solution** at room temperature in the dark.

The blue-purple precipitate became visible after 15-30 min of incubation.

After colour development, the reaction was stopped by washing in 50 ml of milliQ water for 5 min. The blot was then analyzed for positive signal.

**Blocking/Washing buffer 1X (210 ml):** Dilute 21 ml of the concentrated 10X **Blocking/Washing buffer** with 189 ml of milliQ H₂O. Diluted buffer can be stored at 4°C for 1 week.

**Blocking solution (60 ml):** Dissolve 0.6 g of **Blocking reagent** in 60 ml of 1X Blocking/Washing buffer, and stir the suspension on a magnetic stirrer until the Blocking reagent completely dissolves. Shaking of the suspension at 50-60°C facilitates dissolution of the blocking reagent.

**Streptavidin-AP conjugate (30 ml):** Dilute concentrated **Streptavidin-AP conjugate** 5000 fold by adding 6 µl in 29.994 ml of Blocking solution just prior to use.

**Detection buffer (60 ml):** Dilute 6 ml of **10X Detection buffer** in 54 ml of milliQ water. Diluted
buffer can be stored at 4°C for 1 week.

**Substrate solution (30 ml):**

Dilute 600 µl of 50X BCIP/NBT solution with 29.4 ml of 1X Detection buffer. The Substrate solution should be prepared just prior to use.

1.7. **Pre-hybridization buffer (can be stored at RT for 2 months; for 100 ml)**

- Formamide (50% v/v) - 50 ml
- Sodium phosphate buffer (Na₂HPO₄; pH 7.2; 120 mM) - 12 ml (from 0.5 M stock)
- Sodium chloride (NaCl; 250 mM) - 1.461 g
- EDTA (pH 8; 1 mM) - 200 µl (from 0.5 M stock)
- Sodium dodecyl sulphate (SDS 7% w/v) - 7 g

2. **Southern blot hybridization**

2.1. **Restriction digestion of genomic DNA**

- First make the reaction mixture as follows:
  - 10X Buffer (1X) - 5 µl
  - DNA (30 µg) - 10 µl (from 1 µg/µl stock)
  - The specific restriction Enzyme (15 U/µl) - 6 µl
  - MilliQ H₂O to make the final volume

- The above mixture was incubated for 16-18 h at 37°C in a water bath.
- Reaction was stopped by thermal inactivation at specified temperature for 20 min.
- Centrifuged at 10,000 rpm for 1 min.
- Added 2 volumes of chilled ethanol and 1/10 vol. of 3 M sodium acetate and incubated at -80°C for 1 h.
- Centrifuged at 13,000 rpm for 25 min at 4°C.
- The pellet was washed in 70% ethanol, dried and finally dissolved in 20 µl of TE buffer.
The digested product was electrophoresed on 1% agarose gel at 30 volts in cold room to avoid heating effect for 7-8 h.

2.2. Transfer of the digested DNA to a nylon membrane

The gel was captured in a Gel documentation system (Gel Doc™ XR, Bio-Rad Corporation, USA) but DO NOT OVEREXPOSE TO UV LIGHT.

Transferred the gel to a GEPROTRED and de-purinate by soaking it for 10 min in 100 ml of 0.25 N HCl with gentle shaking on a rocker (25 rpm). After exact 10 min the solution was removed and then rinsed with autoclaved milliQ H2O to remove HCl completely.

0.25 N HCl (250 ml):
- 12.1 N HCl - 5.15 ml
- MilliQ H2O to make the volume 250 ml

Added 150 ml of denaturing solution and agitated the gel gently on a rocker (25 rpm) for 1/2 h at room temperature.

Denaturing solution (150 ml):
- 0.4 N NaOH - 2.4 g
- 0.6 M NaCl - 5.25 g

- MilliQ H2O to make the volume 150 ml

After removing the denaturing solution, the membrane was rinse with milliQ H2O and added 150 ml of neutralizing solution and the gel was kept in it for 1/2 h at room temperature on a gel rocker (25 rpm).

Neutralizing solution (150 ml):
- 1.5 M NaCl - 13.14 g
- 0.5 M Tris (pH 7.4) - 75 ml from 1 M stock

- MilliQ H2O to make the volume 150 ml

The positively charged nylon membrane was cut (Amersham Hybond™-XL, GE Healthcare, Sweden) slightly larger (1-2 mm) than the size of the gel and soaked in 50 ml of 2X SSC buffer for 5-10 min (avoid touching the membrane with hands).

Gel tray was placed upside down in another tray containing 20X SSC and place the 3 mm blotting sheet wick above the gel tray.
• The wick was saturated by putting 20X SSC with the help of pipette from the tray. Keep on saturating the wick for some time and avoid any air bubbles.
• Slowly placed 5 mm sheets (3-4 sheets) one by one above the wick and saturated with 20X SSC.
• Now transferred the processed gel from the GEPROTRED tray to this and then placed nylon membrane directly above the gel and saturated with 20X SSC from the tray.
• Added 5 mm sheets one by one along with saturating it with 20X SSC, then placed stacks of sheets of 5 and 3 mm and filter paper followed by a weight of 1/2 Kg.
• The DNA from the gel was transferred to the membrane by capillary blotting. The process was continued overnight (15-18 h).
• The gel was constantly kept wet with 20X SSC buffer during the transfer. The wells were marked with pencil and then the membrane was removed carefully.
• The nylon membrane was then placed within the folds of the Whatmann No. 3 filter paper and cross linked in a UV cross linker (Hoefer UVC 500, Amersham Biosciences Inc., USA) twice for 2 min each.
• Pre-hybridization, hybridization and immunological detection steps were performed as explained above in Sections 1.3-1.7.