SOUTHERN BLOTTING

1. Restriction and Gel electrophoresis of genomic DNA: Digest about 10µg genomic DNA in 50µl reaction volume with 50 u enzymes for 4- 5 h or overnight in the warm oven (or under mineral oil) in correct temperature. Use first 30 u of enzyme, incubate for 1 h, then remaining 20 u, pipette well and incubate for remaining time. Pay attention to enzyme peculiarities. Stop restriction by adding 7µl of Probe buffer (loading dye) and 1 µl of 20% SDS. Heat 5 min at 70 °C and spin briefly. Prepare 0.7% Agarose- Gel (0.4- 1%, according to interesting fragment size) in 1 x TBE-Buffer with 1 µg/ml Ethidiumbromid (gloves!, carcinogenic). Use 12- pocket comb, 1.5 mm of thick. For medium size gel, prepare 110 ml Gel. With sufficient running buffer over lay, load samples (suitable size markers to the left and to the right at different amounts – 7µl and 15µl!) and run for about 400- 700 Vh with a maximum of 80 V. Run overnight is possible (~25 V). Take note of voltage hours.

2. Documentation: Detach a piece of the gel at the lower left end corner, photograph the Gel with the ruler to the left. Expose only shortly to the UV- light (maximum for 1 minute) (wear protective glasses). Clean UV- disk previously with EtOH prior to viewing gel. Take a picture of the gel with the fluorescent ruler.

3. Transfer: Incubate Gel for 30- 40 min in 0.4 M NaOH (on Gel bed). Hybond N+ and 3 sheets of filter paper (Whatman 3 MM or Schleicher and Schuell GB 002) on Gelgröße right cutting edge. Moisten in water, then in **20 X SSC**. Handle membrane only with clean tweezers.
In the construction of the capillary blots, (s. drawing) guarantee that between gel and membrane, no airs bubbles are caught; roll using a glass pipette to get rid of bubbles.
Fundamental construction: Buffer reservoir with 300- 400 ml 20 x SSC - 2 x filter papers - Gel – membrane - 2 x filter paper - pile of paper cloths - glass plate- about 200 g- weights. In the construction, guarantee that paper cloths do not hang in reservoir. Therefore, around the gel strip place Parafilm. For transfer of Plasmid- DNA or lambda- DNA- fragments can be transferred without the reservoir of SSC. A clean glass disk serves as a support for the gel. Transfer at least 12 h for genomic DNA, for alternative method 1- 2 h will be sufficient. After transfer, remove paper cloths and filter paper, mark position of the gel- pockets with soft pencil on membrane. Cut off lower corner of the membrane. Wash membrane for 15 seconds in 2 x SSC and dry totally on filter paper. Fix DNA by baking the membrane at 80°C for 2 h or by UV crosslink DNA for ~30 sec (0.16kJ/m^2).

4. Prehybridization: Cut the membranes according to the probes that it would be hybridized with. Moisten membrane strips in 5 x SSPE and transport into appropriate clean glass tubes. Add 25 ml hybridization buffer incubate for 30 min (or longer – maximum ON) at 42°C. In the meantime, prepare the probe.

5. Labeling of the probe (Fermentas DecaLabel Kit, K0622) Statements are for DNA- probes.
Typically, labelling is done with dCTP. For very hot probes, you can combine dATP and dCTP.
For labelling the probe, use 25 ng DNA.

- add 10µl buffer, fill to 40µl (sigma water)
- Vortex, spin down.
- 10 min in boiling water bath (heating block is not enough), snap cool on ice. The DNA is denatured now.
- Go to the isolab immediately (sample on ice), spin down there.
- Add 3µl MixC (dNTP without dCTP), 5µl radioactive labelled dCTP, 1µl Klenow enzyme.
- Mix, spin down, 5 min 37°C heating block. Now the labelled probe is created, the correct temperature is very important.
- Add 4µl dNTP, another 10 min 37°C. Check temperature!
- Stop reaction with 1µl EDTA (0,5M).
- Add TE to 200 µl, mix carefully.
- Precipitate with 400µl EtOH abs, 40 µl NaAcetate, 1µl glycogen. 15 min(!) 14.000 rpm RT.
- Collect supernatant for Szintillation counter.
- Wash pellet twice with 400 µl of 70% EtOH, spin 2 min. each time at 14.000 rpm, RT, air-dry.
- Resuspend in 50µl Sigma water
- 1µl into counter. A good incorporation rate is above 50%, you need at least 10 Mio counts in probe total.

Calculation: counts supernatant + counts probe = counts total (~ 50-60 Mio in 5µl fresh dCTP). Incorporation rate = counts probe / counts total.

6. Hybridization: Before adding the probes to the hybridization tubes the probe and the amount of salmon sperm DNA (100 µg/ml hybridization solution) need to be denatured. Add the salmon DNA to the E-Tube containing the labeled probe and incubate 5 min at 95°C. Centrifuge briefly for a few

seconds and add the probe to the hybridization tubes (do not add directly on membrane). Incubate at 42°C at least for 16 h (for Oligo- samples shorter time is sufficient; and the buffer conditions are also different)[f].

f Aqueous solution of formamide dentures DNA. Hybridization can take place at lower temperatures (42°C), resulting in lower background, in an increased retension of immobilized DNA. In addition, lower hybridization temperatures allow an easier control of stringency during the hybridization.

7. Washing: After the hybridization, adjust shaking water bath to 65 °C (and/or other temperature). Prepare 1L of washing solutions per membrane: a) 2 x SSC/0.1% SDS and b) 0.2 x SSC/0.1% SDS. Discard the probe (or store it at –20ºC in a lead container for 2-3 immediate uses). Gently place the membrane in plastic tray with 300 ml of 2 x SSC/0.1% SDS. Wash 2 x 15 min at RT. Disgrad washing solution in the radioactive waste. Add pre-warmed (65°C) 0.2 x SSC/0.1% SDS washing solution. Wash 2 x 15 min at 65°C in the shaking waterbath. Discard washing solution in the radioactive waste. Allow the liquid to drip from the membrane and place it in between plastic sheets and seal all the sides. Expose overnight along with StrataLogos. (Check if the X-ray cassette is placed between lanthanide sheets.)

8. Rehybridization: Membrane must not be allowed to dry! First remove probe: wash membrane with 300 ml boiling Buffer (10 mM of Tris- HCl pH 8/ 0.1% SDS) and repeat once after 20 min incubation. Then advance to step 5, 6 and 7. Labeled probes in hybridization puffer needs to be denatured before reuse for 10 min at 80°C and rapidly cool on ice (Use falcon tubes!)

9. Comments: Glass tubes must be cleaned extensively. After each use, rinse with running water for 1 hour and check for radioactivity. Then incubate for 20 min. with 50ml 1 N NaOH, then thoroughly with deionized water (5 x). Under these conditions, they are suited also for the use with RNA. For less stringent hybridization conditions, in hybridize in 37° instead of 42°C. Wash first in 2 x SSPE/ 1% SDS at 42°C, then expose for 1- 2 h. Increase temperature in 5°C- steps, set ions strength first to 0.5 X SSC, then 0.2 and 0.1 X SSC. Control single steps by monitoring and/or through exposing.

Hybridization with Riboprobes:

Moisten the membranes in 5 x SSPE, and then incubate it for at least 30min in 25 ml hybridization buffer (membrane size approx.. 12 x 14 cm) at 65°C in glass tube. Denature the riboprobe for 2 min. at 80°C and cool quickly on ice. Add the probes into the hybridization solution (do not add directly on membrane!) and incubate for 16-20 h at 65°C.

Wash: Wash the membranes in a container as described with NaOH. 3 x 20 min at 70°C in 300 – 400 ml 0.1 x SSPE/0.1 % SDS in a shaking water bath. Allow the solution to drip and while still damp, seal the membrane between polythene sheets and expose for 1-2 hrs or overnight.

RNAse treatment: Incubate membrane for 20-30 min at room temperature in 2 x SSPE/0.1 % SDS and 20 µg/ml RNAse incubate in a shaker. Then, wash 4 x for 2-3 min. in same buffer without RNAse. Finally, wash for 2 x 5 min at 70°C in 0.1 x SSPE/0.1 % SDS, seal moist and expose

- The RNAse treatment increases the specificity of hybridization, however decreases the sensitivity. Therefore, first expose without RNAse. The wash temperature after hybridization can be increased on 75-78°C for better stringency.

- The hybridization and wash temperatures can be lowered for lower stringency to 45-50°C Additionally the ion strength can be increased during washing to 0.2-2 x SSC

- Removal of Ribroprobes: Blot 2 x 20 min for each in 100 ml 0,25 M NaOH, 4 x 5 min in 200 ml 2 X SSPE, wash and dry.

8. Solutions:

10 x TBE:	0.89 M Tris base	54 g
	0.89 M Borsäure	27.5 g
	8 mM EDTA	20 ml aus 0.5 M pH 8- Stocklösung, autoklavieren

10 X Probenpuffer:	30 % Ficoll Typ 400
	0.1 M EDTA
	0.25 % Bromphenolblau
	0.25 % Xylencyanol

0.25 % Orange G

20 X SSC und SSPE: siehe Maniatis oder Laborprotokoll

Hybridisierungspuffer:

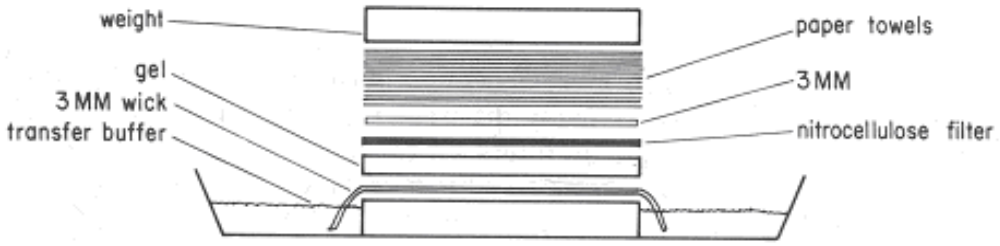
Reagenz	Endkonzentration	125ml
FORMAMIDE *Ultrapure (Fluka)*	50%	62,5 ml
SSPE	6x	37,5 ml
Denhardt (100x)	5x	5 ml
Dextran Sulfate (50%)	10%	12,5 g
SDS	1%	6,25 ml
denat.Lachs/Hering Sperm [10 mg/mL]	100µg/ml	

Denhardt's Solution (100x)

Reagenz	Endkonzentration	50ml
Ficoll 400	2% (w/v)	1 g
Polyvinylpyrrolidone	2% (w/v)	1 g
BSA Fraction V	2% (w/v)	1 g

- Use of formamide allows hybridization at lower temperatures (see above)
- When formamide is included in the hybridization buffer, SSPE is preferred to SSC because of its greater buffering capacity.
- Salmon-DNA needs to be carefully sheared before use! See additional protocol for the preparation of salmon sperm DNA.
- Dextran sulphate enhances the rate of hybridization. However, larger volumes of washing solutions are required to remove it from the membranes after hybridization, in order to avoid background.

Transfer arrangement:



NOTES ON SOUTHERN BLOT HYBRIDIZATIONS

Pretreatment of gel: Soak gel about 30‘ in 0.25M NaOH. If very large DNA fragments are to be transferred (>20kb), soak in 0.25M HCl for 10 min before NaOH treatment.

Transfer/Fixation: Transfer DNA without prior neutralization onto uncharged (if using Digoxigenim-labelled probe) or positively charged Nylon membrane, using 10 x SSC or SSPE. For genomic DNA , allow for >12 hrs, plasmid DNA (≤ 5 kb) transfers efficiently in 1-2 hrs.
After transfer, wash gel briefly in 2 x SSPE. Dry to completion onto 3MM paper. UV crosslink DNA for ~30 sec (0.16kJ/m^2).

Prehybridization: Prewarm prehybr. Solution: 50 % Formamide / 5 x SSPE / 5 x Denhardt / 1 % SDS / 100µg / ml DNA or RNA. Soak membrane briefly in 5 x SSPE, then prehybridize for 30-60 mins (or longer, if convenient) at 37-42°C.

Hybridization: Remove prehybr. Solution, add probe (denatured, if DNA is used) in 25-30ml of hybr. solution (identical to prehybr. Solution for standard conditions, see below). Hybridize at 42°C (stringent) or 37°C (less tringent). ~40 hrs exposure time may be required for detection of single copy genomic sequences.

Washing: Remove (and store) probe. Wash 3 x 30-45 min in 0.1 x SSPE / 0.5 % SDS at 65-70°C (stringent) or at lower temperature if heteroologous sequences are to be detected. Seal membrane and expose or go to non-radioactive detection step.

Notes:

Determination of melting point for perfect DNA-DNA hybrid:

$T_M = 81.5 + 16.6 \log\ ]Na^+] + 0.41(G+ C) - \frac{500}{} - 0.62$ (% Formamide)

L

(L = length of double strand). Hybridization should be carried out at –25°C below T_M. T_M decreases 1°C / 1% mismatch for hybrid >150 bp.

Determination of hybridization time:

T_H = 1:X x Y:5 x Z:10 x 2

X = amount of probe in µg, Y = complexity of probe in kb, Z = hybridization vol in ml.

Multiply x 3 to achieve ~75 % hybridization efficiency.

Determination on melting temperature during washing:

(conditions are ~0.1 x SSPE) : $T_M = 69.3 + 0.41(G+C)$ %

For stringent washes, temperature should be ~5°C below T_M.

Removal of probe:

DNA-DNA: Incubate membrane 2 x 20 min in 0.25M NaOH / 1mM EDTA at 37°C. Neutralize 2 x 5-10 min in 2 x SSPE at room temp.

RNA(probe)-DNA: 100µg/ml RNAse A/T, in 2 x SSPE at 37°C for 30-45 min, followed by three washes 10 min each in 0.1 x SSPE/1 % SDS at 37°C.

Variation of hybridization / washing conditions:

Detection of related sequences in distantly related species:

30 % Formamide / 5 x SSPE / 5 x Denardt / 1 % SDS / 100 µg/ml nucl. acid at 37°C. Washes: Start in 0.1 x SSPE / 0.5 SDS at 37°C, expose, increase temp. In 5°C steps. Some refs. use 2 x SSPE / 0.5 SDS to start at 37°C.