SOUTHERN BLOT

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For any question, please contact:

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1. SAFETY REQUIREMENT

Handle Ethidium bromide gel with gloves
Use protective glasses against UV

USING RADIOISOTOPE P32 SAFELY**:**
Work in a room dedicated to radioactivity
Work behind a protective screen
dosimeter, counter type
Gloves
A full-length lab coat (worn closed with sleeves rolled down)
Close-toed shoes. Do NOT wear sandals or other open-toed shoes while working with radioactivity
Protecting closing

Abbreviation
WT: wild type DNA
BET: Ethidium bromide
ES cells: embryonic stem cells

Notes

The size of the restriction fragments should not exceed 25 kb (the size of larger fragment is impossible to determine on an agarose gel.

2. QUALITY CONTROL

Take a picture of the BET stained gel
Check the correct digestion and quantity of DNA after migration and before transfer

3. BIOLOGICAL MATERIAL

Genomic DNA from ES cells or mouse DNA (tail or other organ)

4. SUPPLIES

4.1 Equipments

Equipment	Vendor
roller for hybridization 40x300mm	Verlabo 2000
hybridization oven	Fisher scientific
heating block	Grant
agitator	Type GFL 1083
generator	Type Bio-Rad
gel tray	
buffer tank	
developer (and developer solution)	Kodak
water bath or oven	
compact orbital shaker	Type Edmund Buhler
pipettes P2-20-200-1000	Type Gilson
hypercassette for autoradiography (including sensitive preflash gun)	
microwave	
UV transilluminator	

4.2 Material

Reagent	Vendor	Reference
Agarose	Seakem LEA	50004L
Ethidium Bromide	Euromedex	EU0070
GeneRuler 10-01	Fermentas	SM0331
Monocut mix lambda	Biolabs	N3019L
Marker high 10 -48,5	Fermentas	SM1351
Whatman 3MMChr		3 030 917
Filter paper 42 x 52 cm	Machery Nagel	300 403 014
Hybond-XL 20x20 cm	Amersham	RPN203S
SDS 20%	MP	SDS20002
Kit Megaprime DNA labeling System dCTP	Amersham	RP N1607
Alpha-P32 dCTP 37MBq (1mCi)	PerkinElmer	NEG513H001MC
NICK columns Sephadex	Amersham	17-0855-02
Rapid Hybridization Buffer	GE Healthcare Amersham	RPN1636
Kodak BioMax MR 30x40 cm	Kodak	8 929 655
Kodak BioMax MR24x 30 cm	Kodak	8 912 560
Restriction enzyme		

4.3 Consumables

Eppendorf 1.5 ml
Eppendorf Safe-Lock 2 ml
Tips
Plastic pipettes
SaranWrap (or similar cling film)
Plastic bag

4.4 Glass-making

Assorted laboratory glassware

4.5 Probes

Neo probe sequence:
ctgcaggacgaggcagcgcggctatcgtggctggccacgacgggcgttccttgcgcagctgtgctcgacgttgtcactgaagcggga
agggactggctgctattgggcgaagtgccggggcaggatctcctgtcatctcaccttgctcctgccgagaaagtatccatcatggctga
tgcaatgcggcggctgcatacgcttgatccggctacctgcccattcgaccaccaagcgaaacatcgcatcgagcgagcacgtactcg
gatggaagccggtcttgtcgatcaggatgatctggacgaagagcatcaggggctcgcgccagccgaactgttcgccaggctcaagg
cgcgcatgcccgacggcgaggatctcgtcgtgacccatggcgatgcctgcttgccgaatatcatggtggaaaatggccgcttttctgg
attcatcgactgtggccggctgggtgtggcggaccgctatcaggacatagcgttggctacccgtgatattgctgaagagcttggcggc
gaatgggctgaccgcttcctcgtgctttacggtatcgccgctcccgattcgcagcgcatcgccttctatcgc

LacZ probe sequence:
ggcgttacccaacttaatcgccttgcagcacatccccctttcgccagctggcgtaatagcgaagaggcccgcaccgatcgcccttccca
acagttgcgcagcctgaatggcgaatggcgctttgcctggtttccggcaccagaagcggtgccggaaagctggctggagtgcgatct
tcctgaggccgatactgtcgtcgtcccctcaaactggcagatgcacggttacgatgcgcccatctacaccaacgtgacctatcccattac
ggtcaatccgccgtttgttcccacggagaatccgacgggttgttactcgctcacatttaatgttgatgaaagctggctacaggaaggcc
agacgcgaattatttttgatggcgttaactcggcgtttcatctgtgg

4.6 Solutions

Initial concentration: stock solution or product

TAE 50X	Initial concentration	Volume/ quantity
2 M Tris base	powder	242 g
1M Acetic acid	Glacial acetic acid l(17.4 M)	57.1 ml
100 mM Na2EDTA	powder	37.2 g
H2O		qsp 1l

TE pH7.5 or 8	Initial concentration	Volume/ quantity
10 mM Tris HCl	1 M	10 ml
1mM EDTA	0.5 M	2 ml
H2O		qsp 1l

Loading buffer x 6	Initial concentration	Volume/ quantity
Xylene cyanol	powder	125 mg
Bromophenol blue	powder	125 mg
30% Glycerol	99.5 glycerol bidistilled	15 ml
TE pH7,6		qsp 50 ml

Ethidium bromide	Initial concentration	Volume/ quantity
1 mg/ml	10 mg/ml	1 ml
H2O	/	qsp 10 ml

HCl 0.25M (depurination)	Initial concentration	Volume/ quantity
0.25 M HCl	HCl 37% Prolabo(12M)	20,8 ml
H2O	/	qsp 1l

Solution denaturation	Initial concentration	Volume/ quantity
0,5 M NaOH	powder	40g
1,5 M NaCl	powder	175,32g
H2O	/	qsp 2l

20 x SSC	Initial concentration	Volume/ quantity
3 M NaCl	powder	175,32g
300mM Na Citrate	powder	88,2g
H2O	/	qsp 1l

Wash solution 2xSSC; 0.1% SDS	Initial concentration	Volume/ quantity
2X SSC	20X SSC	100 ml
0.1% SDS	SDS 20%	5 ml
H2O	/	qsp 1 l

Wash solution 0.1x SSC; 0.1% SDS	Initial concentration	Volume/ quantity
0.1X SSC	20X SSC	5 ml
0.1% SDS	SDS 20%	5 ml
H2O	/	qsp 1 l

5. OPERATING MODE

5.1. DNA digestion

Digest 20 µg DNA (phenol/chloroform extraction, clean DNA is very important!)
Include a WT DNA control for external probe
Digest in 60 µl final volume (40 µl reaction mix + 20 µl DNA)

Preparation of reaction mix:

Number of reactions	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Buffer10X	6	12	18	24	30	36	42	48	54	60	66
Spermidine 0,1M	1	2	3	4	5	6	7	8	9	10	15
enzyme	5	10	15	20	25	30	35	40	45	50	55
H_2O	28	56	84	112	140	168	196	224	252	280	308
Final volume	40	80	120	160	200	240	280	320	360	400	440

Buffer and incubation temperature as indicated per manufacturer.
Incubation 5 hours or overnight at 37°C (or as specified by the enzyme supplier)
Add 5µl of loading buffer

5.2. Migration of digested DNA

5.2.1 AGAROSE GEL PRÉPARATION

Neo Probe: Gel 0.65% (30 wells) for a 20 X 20 cm tray
Agarose 3.6g + 550 ml TAE 1X + 5 drops Ethidium Bromide at 1mg/ml

External Probe: Gel 0.60% (30 wells) for 20 X 20 cm tray,
Agarose 3.3 g+ 550 ml TAE 1X + 5 drops Ethidium Bromide at 1mg/ml

In the case of large size fragments (over 15 kb) with small differences between WT and targeted bands, lower gel concentration up to 0.5% can be used.

Agarose 2.75 g + 550 ml TAE 1X + 5 drops Ethidium Bromide at 1mg/ml

5.2.2. LADDERS PREPARATION

Reconstitution of ladder GeneRuler DNA ladder SM0331 and SM1351 at 50ng/µl:
100µl of DNA Stock + 750µl H2O + 150µl of loading buffer x6

Ladder neo probe 0.1- >24 kb	Per well 30 µl	20 wells 600 µl
GeneRuler DNA ladder SM0331 reconstituted	3	60
Monocut lambda New England Biolabs 0.5µg/µl	1	20
Loading buffer 1x in water	26	520

Ladder external probe 0.1- >24 kb	Per well 30 µl	20 wells 600 µl
GeneRuler DNA ladder SM0331 reconstituted	10	200
Monocut lambda New England Biolabs 0.5µg/µl	1	20
Loading buffer 1x in water	19	380

Ladder High molecular weight (10 to 48 kb)	Per well 30 µl	20 wells 600 µl
GeneRuler High Range DNA SM1351 reconstituted	10	200
Loading buffer 1x in water	20	400

5.2.3. LOADING AND GEL MIGRATION

Several projects (needing different probes) can be loaded on a same gel; in this case ladders must be loaded of both sides from samples

Migration in TAE 1x

After digestion, load **all** the DNA samples.

Load 30 µl of mix of molecular weight marker on both sides.

Day migration: Start 90 V then 100-110V
Migration overnight: 55V with 1 comb, 25V with 2 combs

After migration, take a gel picture, and mark ladder bands with a small scalpel notch on the gel.

5.3. Alkalin transfer

Keep gel on agarose tray, and place it in a washing tray, on an agitating platform for the next 2 steps.

5.3.1 DEPURINATION (FACILITATES TRANSFER FOR LARGE SIZE FRAGMENTS >10KB)

Incubate gel 15' in 450 ml 0.25 M HCl (loading buffer becomes yellow) and rinse gel with distilled water.

5.3.2. DENATURATION

Exchange water against 450 ml 0.5 M NaOH; 1.5 M NaCl and leave 30' to 45' (loading buffer regains initial color).

5.3.3. TRANSFER

Place in the following order:
2 to 3 packs of paper towels
1 quarter of Whatman 3MMChr leaf
Membrane Hybond-XL cut 20x20 cm
Gel
Saran-type transparent film
Agarose gel tray to apply a light pressure

Allow the transfer to proceed overnight

Before removing the gel annotate the membrane:
Label ladder bands and wells with a pencil through the notch (made at step 8.2.3)
Split each probe with a scalpel and immerge in distilled water.

5.4. Probe labeling

Kit Megaprime DNA labeling System dCTP Amersham

Pre-heat a water bath to 100°C.

Primer NIF541	5 µl
H2O	27µl
Probe 25 ng/µl)	1 µl

5' in a boiling water bath.
Spin briefly to bring the contents to the bottom of the tube
On ice add:

Labeling buffer	10 µl
Klenow	2µl

Keep on ice.

In radioactive area:
Add 5 µl Alpha-P32 dCTP (Total volume: 50 µl)
Incubate 30 to 45 minutes at 37°C
The probe can be immediately purified or stored at -20°C until purification.

5.5. Probe purification

NICK columns Sephadex, Amersham

Remove the top cap of the NICK column and pour off the excess of liquid
Rinse the top of the column once with equilibration buffer and pour away.
Remove the bottom cap.
Equilibrate column with 3 ml TE pH7.5-8.0.
Place column on a 2ml Eppendorf Safe-Lock tube
Add the sample (50 µl of labeled probe) to the top-center of the resin
Add 400µl TE pH 7.5-8.0 and let flow through.
Place column on a new 2ml Safe -Lock tube.
Elute the purified probe with 400µl TE 7.5-8.0
Check labeling with a Geiger counter (for example Canbera MC21): signal must saturate the scale of Geiger counter
Keep the purified probe at -20°C or at +4°C (if you use it the same day).

5.6. Pre- hybridization and hybridization

Plan for each probe: 10 ml Rapid Hybridization Buffer + 300 µl salmon sperm DNA (stock solution at 10mg/ml).
Pre-heat hybridization oven at 65°C; pre-heat to 65°C Rapid Hybridization Buffer.

Denature salmon sperm by heating 5' at 100°C; place it on ice.

Prepare mix with hybridization buffer + salmon sperm as described

AVOID TOUCHING MEMBRANES.

USE ONLY no-powdered gloves.
Transfer membranes (wells at the bottom of the tube and DNA side facing inwards) to a roller containing water or directly in 10 ml hybridization buffer + salmon sperm preheated to 65°C.

Incubate 20' to 1h at 65°C in hybridization oven.

Thaw radioactive probe if necessary; denature in heating block 5' at 95°C (place a weight on the tube if it is not Safe lock); transfer denaturized probe in roller without splashing the membrane.

Hybridize 3h (or overnight) in hybridization oven.

5.7. Washes

Pre-heat washing solutions to 65°C.

Empty rollers into an appropriate recipient.

Rinse with approximately 10 ml 2xSSC; 0.1% SDS; pour it away.

First wash: add approximately 10 ml 2xSSC; 0.1% SDS; let it turn 5' in hybridization oven; pour it away.

Second wash: 10 ml 2xSSC; 0.1% SDS; 15' at 65°C.

Take out membranes:
shake rollers so membrane slides towards the top of the tube; empty washing solution, grab membrane with forceps, and place in a tray containing 0.1x SSC; 0.1% SDS solution.

Wash twice 10’ in a tray, under agitation at 65°C, check membrane with a counter (for example Canbera MC21), signal must be slightly audible. If not, change 0.1xSSC; 0.1%SDS and wash again 15’

Place membranes on paper towels;

5.8. Autorad exposure and development

Place the membrane in a plastic bag cut to dimensions of an autorad film.
Fix membrane in plastic bag with tape; place and fixe with tape the plastic bag in a cassette with 2 screens.

In the dark room, place an autorad film (Kodak BioMax MR 30x40 cm or 24x 30 cm) shiny face upwards; mark film orientation at a corner.

Store cassette at -80°C for 48hrs (adapt time and temperature to radioactive signal intensity after washes)

Bring autorad containing cassettes back to room temperature and develop autorads.
Identify the ladder bands and slots on the film.

5.9. Results for homologous recombination

Neo probes

One band is expected; more than one band will indicate:
- Multiple integrations (to confirm with other digests)
- Star activity or partial digestion

Check conformity of band sizes.

External probes

Interpretation is always compared with the Wild Type (WT) sample.
Two bands of equal intensity are normally expected:
- WT band
- Targeted band

If presence of non-specific bands in the WT sample, the same non specific band should appear in all samples.
In in the case of external probes, if the chromosome X is targeted, only one band corresponding to the targeted allele should be visible (in the case of southern performed on male ES cells)

Check conformity of bands size for the WT and targeted bands. The control is always the WT fragment, even if the size doesn’t fit with the one found in the databases (single nucleotide modification)

Validation of genomic probe

Only one band should appear, at the expected size without background.

In some cases background, non-specific bands can appear (repeated genomic region); the expected size could be slightly different: validation of the probe is to discuss.

5.10. De-hybridization (if needed)

Boil 500ml of bi-distilled water.
Add 2.5 ml SDS 20% (0,1 %SDS).
Put membrane to de-hybridize and agitate for 5-10 minutes.
Repeat and rinse membrane with bi-distilled water.
Dry and keep at room temperature.
Pre-hybridize and hybridize as usual.