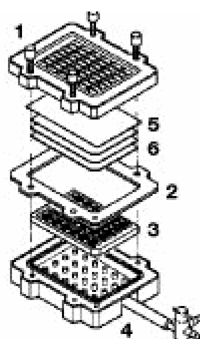
Transcription run-on

(submitted by Bernhard Dichtl and Joanna Kufel)

BLOTTING: SLOT-BLOT – modified from Birse et al., 1997, EMBO J, 16(12) 3633-3643. Modified in Bernhard Dichtl's and Joanna Kulfel's groups, modifications include utilisation of 70-80 nucleotide long oligonucleotide probes complementary to regions of interest instead of single-stranded phage M13-based probes to provide more precise coverage of the transcripts and to simplify the procedure for multiple analyses. In addition, denaturation step was omitted in the hybridisation procedure and Perfect Hyb (Sigma) hybridisation buffer was used.

1. Use BIO-DOT SF (BIO-RAD) apparatus
2. Hybond-XL membrane (Amersham)
3. Pre-wet 3 filter papers (BIO-RAD filter paper, Cat. no. 162-0161) in 5 x SSPE
4. Pre-wet membrane in 5 x SSPE (10 min)
5. Assemble in BIO-DOT SF (tighten the screws using a diagonal crossing pattern to ensure an even pressure on the membrane surface)

1 – sample template with attached sealing screws; 5- membrane; 6-filter papers (3-4 sheets);
2-sealing gasket; 3-gasket support plate; 4-vacuum manifold.



6. Turn on the vacuum and repeat the tightening process.
7. Tightening while vacuum is applied, insures a tight seal, preventing cross contamination between blots.
8. (Turn on the vacuum) rinse wells once or twice with 500 µl 5 x SSPE
   keep on the vacuum
9. Sample preparation: dilute 10 µg DNA in 250 µl 5 x SSPE per well
   keep on the vacuum
10. Apply DNA to wells
11. Rinse wells once with 500 µl 5 x SSPE
12. Unscrew and remove membrane from BIO-DOT with vacuum still on
13. (Denature DNA by immersing membrane in denaturing solution for 5 min)
14. (Neutralise by immersing membrane in neutralizing solution for 2 min)

Points 13-14 OMITTED when DNA oligonucleotide probes (70-80 mer) are used

15. Air dry filter
16. Fix DNA to filter by UV crosslinking once at 120.000 µj/cm^2
17. Prehybridise the filters (see prehybridisation section)

RUN-ON

1. 100 ml cultures were grown in complex or SD media based on YNB (Yeast Nitrogen Base), in various C sources (galactose, glucose, ethanol or raffinose), to an OD_{600} of approximately 0.10-0.20 at appropriate temperature, usually room temp. or 30ºC.
2. When cells reach correct OD: 0.10-0.20, harvest culture in 50 ml Falcon tubes, spin setting 3500 rpm on bench-top centrifuge for 2-3 min at 4ºC.
3. Remove supernatant, wash cells in 5 ml ice cold dH_2O
4. Resuspend cells in 1.2 ml ice cold dH_2O, transfer to an Eppendorf, remove 0.25 ml (for steady state analysis, freeze in EtOH/dry ice bath)
5. To remaining 0.95 ml add 50 µl sodium N-lauryl sarcosyne sulphate (10% Sarkosyl), mix by inverting tubes 5-6 times, leave on ice 20 min

   During this time, transfer UTP, DTT and rNTPs from freezer to bench. Get hold of screen, counter.
6. After 20 min permeabilisation, spin for 1 min at 8000 rpm in table top centrifuge, remove supernatant, repeat.

In vitro transcription

1. Resuspend cell pellet in transcription buffer

Pre-warmed at 30ºC	2.5x transcription buffer	60.0 µl
	rNTP's ACG mix (10 mM each)	8.0 µl
	DTT (100 mM)	3.0 µl
	H_20 to final volume of appr. 147 µl	

[2.5x transcription buffer (50mM Tris/HCl pH 7.7; 500mM KCl; 80mM $MgCl_2$)]

2. Transcription is allowed to proceed for 5 min at 30ºC following the addition of α-UTP, (60-120 µCi for each transcription reaction).

3. Cells are then washed briefly with 1 ml water before total RNA is extracted. Collect supernatant and discard in radioactive waste. Proceed to RNA extraction.

Yeast RNA Extraction – small scale (Eppendorf tube) (Martin Kos; Tollervey lab)
RNA extraction method is changed to the protocol of Martin Kos. RNA is extracted by GTC/phenol mix using zirconia-silica beads to break the cells.

1. Add 100 µl GTC/phenol mix
   100 µl glass/zirconia-silica beads
   Vortex for 5 minutes on a multitube vortexer at 4°C room.
2. Spin 1 s ! (to get all the liquid from the lid down)
   Add 700 µl GTC/phenol mix
   Vortex and incubate at 65°C for 5 min.
3. Spin 1 s !
   Add 350 µl Chloroform
   120 µl 3M NaOAc (pH 5.2)
   Vortex for 20 s
   Spin 5 min, room temperature
4. Transfer 550 µl of the upper phase into a new tube
   add 500 µl phenol/chloroform
   Vortex 20 s and spin 5 min, RT
5. Transfer 500 µl upper phase into a new tube
   and extract with 500 ml chloroform.
6. Transfer 450 µl of the upper phase
   add 1 ml 100% Ethanol (-20°C)
   Incubate on ice for 10 minutes.
7. Spin 10 min, 20000g, 4°C.
8. Wash pellet with 70% EtOH
9. Resuspend in 50 µl water.

GTC mix
100g Guanidine Thiocyanate (Sigma G9277)
100 ml H_2O
10.6 ml 1M Tris-HCl pH 8.0
4.24 ml 0.5M EDTA pH 8.0

Dissolve 10 min at 60 – 70 °C
Add 2.1 ml β-mercaptoethanol
21.2 ml 20% sarkosyl

GTC/Phenol mix 1:1 with phenol for RNA

Hybridisation

1. Resuspend pellet in 40 µl dH_2O, use new sterile bottle
2. Partial RNA hydrolysis with 10 µl of 1.0 M NaOH for 5 min on ice (0.2 M final conc.)
3. Neutralisation with 20µl 0.5 M Tris / 0.5 M HCl.
4. Add ~ 650 µl of prehybridisation buffer

Prehybridisation

1. Prehybridise the filters in a large volume of prehybridisation buffer for 6-8 hr at 42ºC
2. Remove the prehyb.buffer and transfer hot RNA in the tube
3. Hybridise O/N at 42ºC
   (perform hybridisation in a 15ml Falcon)

Washing filters

1. Wash filters in a large volume of buffer appr. 400 ml to minimise/avoid washing effects
2. 1st wash: 15 min 2x SSC, 0.1% SDS, room temperature.
   2nd wash: 15 min 0.2x SSC, 0.1% SDS, room temperature
   3rd wash (not for oligo probes): 15 min 0.2x SSC, 0.1% SDS, 37ºC;
   Phosphorimage O/N

When using oligonucleotide probes Perfect Hyb (Sigma) solution is used for pre-hybridisation and hybridisation.

Pre-hybridisation solution (make fresh)

	30 ml	50 ml
SSC 20x	7.5	12.5
SDS 10%	0.6	1.0
Denhardt's (50x)	6.0	10.0
dH2O	0.9	1.5
Formamide	15.0	25.0

Filter sterilise

TES

TrisHCl (pH 7.5)	10 mM
EDTA	10 mM
SDS	0.5%
Filter sterilise	

Denhardt's solution (50x)

Ficoll	5g
Polyvinylpyrrolidone	5g
BSA	5g
H_2O	to 500ml
Filter sterilise	

SSPE (20x)

NaCl	3.6M	212g/l
$Na_2HPO_4.7H_2O$	0.2M	31g/l
$Na_2EDTA.2H_2O$	20mM	7.4g/l

Dissolve 210.0g NaCl, 53.6g $Na_2HPO_4.7H_2O$, 7.44 g EDTA in ddH_20, by heating and titrate with 10M NaOH (or NaOH pellets ~5g/l) to pH 7.4
Autoclave

Denaturing solution

NaCl	1.5 M
NaOH	0.5 M

Neutralising solution

NaCl	1.5 M
TrisHCl, pH 7.2	0.5 M
EDTA	1 mM

SSC 20x

NaCl	175.3 g
Sodium citrate	88,2 g
H_2O	800 ml

Adjust the pH to 7.0 with NaOH (few drops)
Adjust the volume to 1 liter with H_2O
Sterilize by autoclaving