

Yeast Genome Arrays

- Protocols and Spot Organisation -

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Mechanical disruption of yeast cells

1. Grow yeast cells to the desired growth rate. A OD_{600} of 0.5 is recommended for best results of cell lysis, but higher densities are also possible.
2. Harvest the cells by centrifugation at 3000rpm for 3 min.
3. Resuspend in a very small volume of growth medium.
4. The suspension is sucked into a pipette and released as individual drops directly into liquid nitrogen.
5. Frozen cell drops can be stored at $-80^{\circ}C$.
6. Pre-cool a 5ml Teflon vessel in liquid nitrogen.
7. Add a 7 mm bead made of tungsten carbide and frozen cells equivalent to about 15 OD_{600} units of cells.
8. Close the flask with the precooled cap and place it into the holder of the micro-Dismembrator (Braun, Melsungen)
9. Set the shaking frequency to 2600 rpm and the operation time to 2 min and start.
10. Take up the still frozen powder in the reagent for RNA isolation (1ml TriStar ReagentTM/ OD_{600} unit)

Isolation of total RNA (TriStar ReagentTM, AGS)

1. Homogenize by vortexing for 1 min
2. Keep samples for 5 min at room temperature to allow for dissociation of the nucleoprotein complexes
3. (Transfer to a Correx tube and centrifuge 12000xg, 10 min, $4^{\circ}C$, transfer clear supernatant to a fresh falcon tube; optional step to precipitate polysaccharides, extracellular membranes and high molecular weight DNA)
4. After addition of 2/10 volume chloroform shake the samples by hand vigorously for 15 sec and keep them for 3-10 min at room temperature.
5. Centrifuge at 12000xg for 5 min. The mixture separates into the lower red (phenol-chloroform phase), the interphase and the colorless upper aqueous phase. RNA is forced exclusively into the aqueous phase whereas DNA and the proteins partition into the interphase and lower phenol phase.
6. Transfer the aqueous phase to a fresh tube (The volume of the RNA containing phase is about 60 % of the volume of the reagent used for homogenization).
7. Precipitate the RNA with 0.5 ml of isopropanol per 1 ml of reagent and keep samples at room temperature for 5-15 min

8. Centrifuge for 10 min at 4°C at 12000xg. The RNA pellet should form a gel like precipitate on the bottom and the side of the tube
9. Remove the isopropanol supernatant carefully
10. Wash the pellet twice with 1 ml of 70% EtOH by vortexing and centrifuge as above
11. Briefly dry the RNA pellet by air-drying
12. Dissolve the RNA pellet in 500 µl ddH₂O (DEPC treated) and transfer to a 1.5ml tube
13. Add 1 volume LiCl-buffer and precipitate for at least 1 h at -20°C.
14. Centrifuge at max speed for 30 min at 4°C
15. Wash the pellet twice with 70% EtOH (salts are inhibitors of Reverse Transcriptase)
16. Dry pellet and dissolve in a small volume of DEPC treated ddH₂O

Buffers and solutions:

TriStar Reagent™ (AGS)

Chloroform

Isopropanol

70% Ethanol

ddH₂O (DEPC treated)

LiCl-buffer: 4 M LiCl

20 mM Tris/HCl pH7.5

10 mM EDTA

First strand cDNA synthesis

1. The equivalent of 20-30 μg total RNA is dissolved in 12.5 μl DEPC- H_2O
2. Add 1 μl oligo dT₁₅ (500 ng).
3. Incubate at 70 °C for 10 minutes. Cool briefly on ice.
4. Place at 42 °C.
5. Mix and add :

6 μl	5x First Strand Buffer
3 μl	0.1 M DTT
<u>1.5 μl</u>	AGT+C mix
10.5 μl	final volume
6. Add

5 μl	³³ P α -dCTP (50 μCi)
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7. Add

<u>1 μl</u>	SuperScript RT (200U)
30 μl	final volume
8. Incubate at 42 °C for 1 hour.
9. Take out 1 μl to check the incorporation of radioactivity. See protocol for "Precipitation of nucleic acids with TCA".

Buffers and solutions:DEPC-treated H_2O Oligo dT₁₅ (500 ng/ μl)

5x First Strand Buffer (Gibco BRL):	250 mM	Tris/HCl pH 8.3
	375 mM	KCl
	15 mM	MgCl ₂

0.1 M DTT (Gibco BRL)

AGT+Cmix	16 mM	dATP
	16 mM	dGTP
	16 mM	dTTP
	100 μM	dCTP

[α -³³P]-dCTP (2500 Ci/mmol) (Amersham)SuperScript RT (200U/ μl) (Gibco BRL)

Alkaline hydrolysis of RNA

1. Add 1 μ l 1% SDS
 1 μ l 0.5 M EDTA pH 8.0
 3 μ l 3 M NaOH
2. Incubate at 65 °C for 30 minutes.
3. Incubate at room temperature for 15 minutes.
4. Add 10 μ l 1M Tris/HCl pH 8.0
 3 μ l 2N HCl

Buffers and solutions:

1 % SDS
0.5 M EDTA pH 8.0
3 M NaOH
1 M Tris/HCl pH 8.0
2 N HCl

Isopropanol precipitation

1. Add 5 μ l NaOAc pH 5.3
 5 μ l tRNA 10 mg/ml
 60 μ l isopropanol
2. Precipitate at -20 °C for 30 minutes.
3. Centrifuge at maximum speed for 30 minutes.
4. Redissolve the cDNA in 100 μ l ddH₂O.
5. (Alternatively use a QIAquick Nucleotide Removal Kit.)

Buffers and solutions:

3 M NaOAc pH 5.3
tRNA (10 mg/ml)
isopropanol

Hybridisation with complex probe

1. Pre-hybridise the filter for 2 hours at 65 °C with hybridisation solution.
2. Denature the probe by adding 1/10 volume 3 M NaOH (or by incubating 5 minutes at 100°C).
3. Hybridise overnight at 65 °C with the probe mixed in 5 ml pre-heated hybridisation solution.

Buffers and solutions:

Hybridisation solution	5x	SSC	
	5x	Denhardt's	
	0.5 %	SDS	
20 x SSC:	3 M	NaCl	
	0.3 M	Na ₃ Citrate	
100 x Denhardt's:	2 % (w/v)	BSA (bovine serume albumine)	
	2 % (w/v)	Ficoll™	
	2 % (w/v)	PVP (polyvinylpyrrolidone)	

Washing the filter

1. Wash briefly with wash buffer I.
2. Wash 20 minutes at 65 °C with wash buffer I.
3. Wash 60 minutes at 65 °C with wash buffer II.

Wash buffers

- | | | |
|-----|-------|-----|
| I. | 2x | SSC |
| | 0.1 % | SDS |
| II. | 0.2x | SSC |
| | 0.1 % | SDS |

Exposure

1. Drain the membran but avoid drying.
2. Wrap the film in saran wrap carefully, make sure that the wrap is as flat as possible (avoid air bubbles since they will decrease the signal).
3. Expose for 1-3 days on an imaging plate (IP).

Regeneration of the filter

1. Boil 300 ml of the stripping buffer.
2. Pour 100 ml of the solution directly onto the filter. Cast away immediately and repeat.
3. Pour the rest onto the filter and allow to cool to room temperature.

Stripping buffer

5 mM Sodium phosphate buffer pH 7-7.5

0.1 % SDS

Precipitation of nucleic acids with TCA

1. Take 1 μ l of the sample.
2. Mix it with
7 μ l 0.05 M EDTA pH 8.0
7 μ l ddH₂O
5 μ l tRNA (10 mg/ml)
3. Put 10 μ l aliquots on the centre of two separate pieces of GF-F Whatman paper (approx. 1 cm ϕ). Mark them differently for instance by cutting the corners in various ways. One filter is kept for measuring the total amount of radioactivity (*total filter*). The other is to measure only the acid-precipitable radioactivity (*TCA filter*). Under the conditions described, DNA and RNA molecules more than 50 nucleotides will be precipitated on the surface of the filter.
4. Let them dry completely at room temperature.
5. Soak the *TCA filter* in a beaker containing ice-cold 10% TCA and 1% sodium pyrophosphate for 5 minutes. Swirl the beaker from time to time.
6. Discard the liquid and repeat twice.
7. Wash this filter in 96% ethanol at room temperature for 2 minutes.
8. Let them dry completely.
9. Insert each of the filters into a scintillation vial and add the scintillation fluid, to enhance the efficiency of the measurement of the radioactivity.
10. Measure the ³³P with an appropriate program of the liquid scintillation counter.
11. % incorporation = counts on "*TCA filter*" / counts on "*total filter*".

Materials:

0.05 M EDTA pH 8.0

tRNA 10 mg/ml

GF-F Whatman paper

Precipitation sol.: 10% TCA (Trichloroacetic acid)
1% sodium pyrophosphate100 % TCA: 500 g TCA and 227 ml H₂O; keep in a dark bottle.

96% ethanol

Scintillation cocktail

Organisation of the spot positions on the filters

- The arrays are divided into three fields, 1, 2 and 3 (fig.A.).
- Each field has 16 rows (A-P) and 24 columns (1-24), forming 384 blocks (fig. A).
- Each block, defined by row and column coordinates, consists of $4 \times 4 = 16$ spots (fig.B).
- Each gene is represented twice (a and b) and the internal positioning of these two spots is one of eight different 4×4 pattern (raster spot positions) (fig.B, C).
- To be able to discern the orientation of the membrane, different positions have been left empty in the three fields (fig D).
- 18 plates have been deposited in the three fields
 - field 1 plates 1-6
 - field 2 plates 7-12
 - field 3 plates 13-18
- Each gene is thus designated by :

PLATE	:	ROW	:	COLUMN	
001-018 a		A-P		01-24	(primary spot)
001-018 b		A-P		01-24	(secondary spot)

For example:

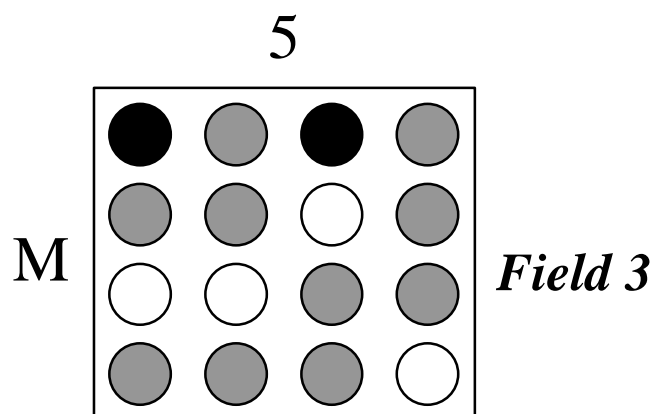


Plate no.: 14
 Row: M
 Column: 5

The two black spots have the designation: 14a M 5 ; 14b M 5

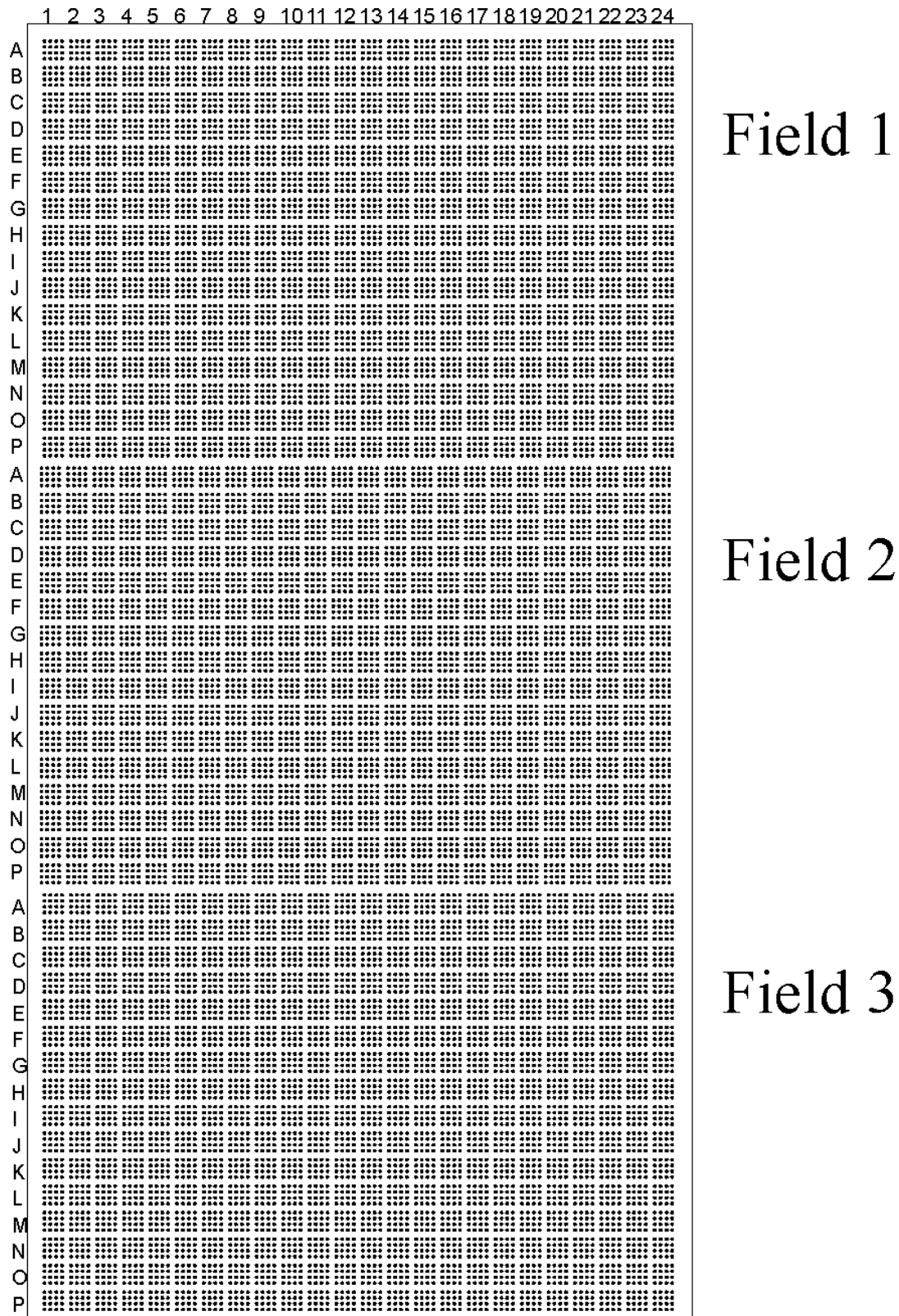
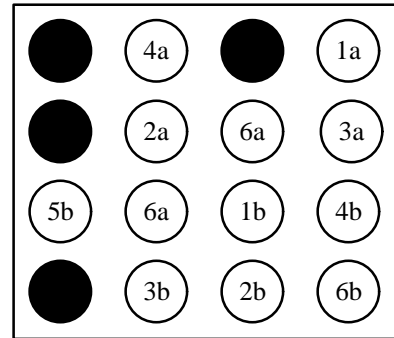


FIG A Organisation of the spots on the arrays

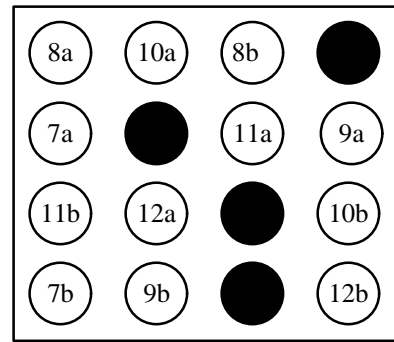
Field 1

Plates 1 – 6 have been deposited twice (a, b)



Field 2

Plates 7 – 12 have been deposited twice (a, b)



Field 3

Plates 13 – 18 have been deposited twice (a, b)

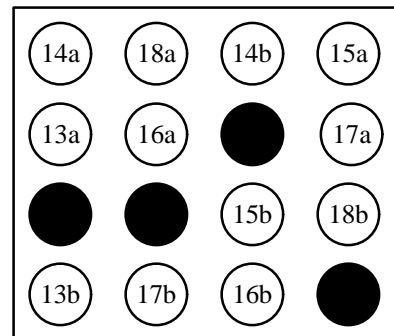


FIG B Close up of the areas designated by a coordinate in the three fields

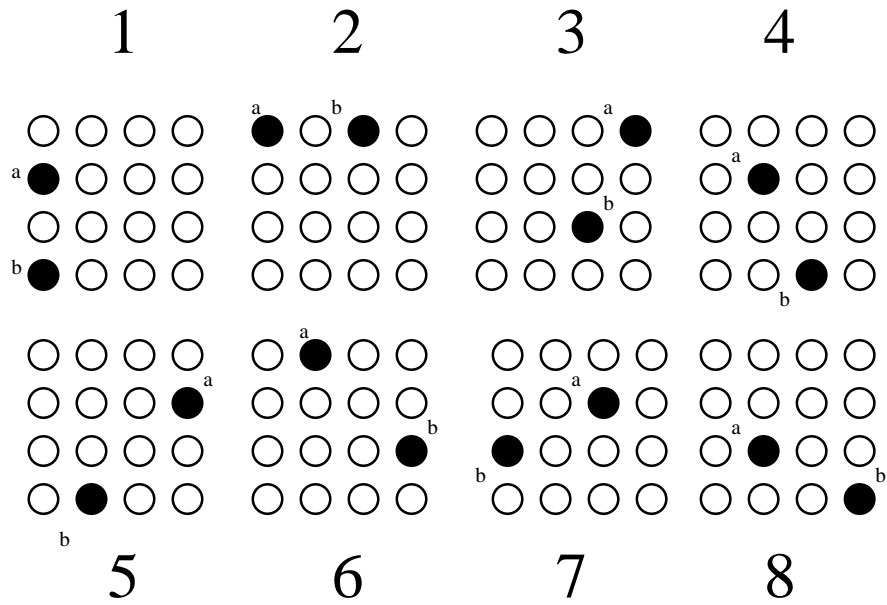
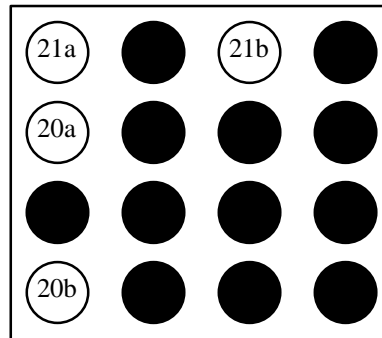


FIG C Eight possible positions, where eight different plates can be spotted in a double offset (a and b)

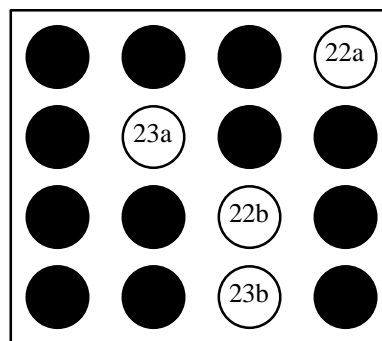
Field 1

Positions 1 and 2 are left empty



Field 2

Positions 3 and 4 are left empty



Field 3

Positions 7 and 8 are left empty

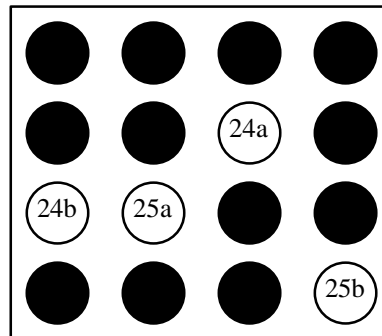


FIG D To be able to distinguish between the top and bottom of the membrane, defined positions have been left empty in the three different fields. Designated spots can be used for local background correction within each block