***S. pombe* 2D gel analysis** **E. Noguchi**

Reagents for DNA preparation

NIB (Nuclear Isolation Buffer) : 500mL

17% glycerol	60%	142mL
50mM MOPS buffer (m.w.:209.3)		5.23g
150mM potassium acetate	1M	75mL
2mM magnesium chloride	1M	1mL
500uM spermidine trihydorochloride (Sigma S-2501)(m.w.:254.6)		63.7mg
150uM spermine tetrahydrochloride (Sigma S-1141)(m.w.:348.2)		26.1mg
		H2O to 500mL

adjust pH to 7.2 after all ingredients are dissolved (by KOH)
keep at 4C

20mg/ml proteinaseK in H2O (500ul aliquot stored at -20˚C)
70% EtOH
Zymolyase 100T
Sarcosyl NL30 (N-Lauroyl sarcosine sodium salt, ICN, Cat 190289)

1. Cell preparation
Grow cells (~ 5 x 10^6 cells ; 400mL : about 200 OD cells)

Transfer culture to 500mL centrifuge tube

Add sodium azide to culture (0.1% final from 10% stock solution)

Chill on ice for 5min

Collect cells by centrifugation (5000rpm, 5min, 4C, Beckman JA-10)

Wash cells with 30mL of H2O (ice-cold)

Transfer to 50mL conical tube (label tubes on the cap and side close to the top)

Collect cells by centrifugation (2000rpm 2min, 4C, table-top)

Freeze cell pellet in EtOH-dry ice (make sure labels are not wiped off) or in liquid nitrogen

Store cell pellet at -80˚C

2. DNA preparation *(*Modified version of *Segurado et al., 2002, Mol Cell, 10, 907-916)*

Day1:
Thaw cell pellet on ice

Prepare NIB containing 2mg/ml of Zymolyase (Zymolyase may not completely dissolved. Use as suspension)

Add 2.5 ml of NIB (ice-cold)

Add 2.5 ml of NIB containing 2mg/ml of Zymolyase

Incubate at 37°C for 20min (mix by swirling tube every 5min)
Check cell lysis: (A) 3ul of cell suspension + 3ul of 2% SDS
(B) 3ul of cell suspension + 3ul of water

Compare A and B. More than 80% of cells should be broken in A
If not, incubate longer and check cell lysis again.

Add 30ml of water to lyse cells.

Transfer broken cells to 50ml round bottom polypropylene tube

Centrifuge (10min 4°C, Beckman JA-17: 6500 x g)

Re-suspend pellet (nuclei) gently by pipetting up and down in 3 ml of TEN (if the pellet is too tight, mash the pellet by glass rod then suspend by pipetting) (DO NOT VORTEX)

Prepare 14ml round bottom polypropylene tube with 45 mg of Sarcosyl NL30 (final 1.5%)

Transfer the suspension to the 14ml round bottom polypropylene tube

Mix well by inverting tubes (DO NOT VORTEX)

Add 48 ul of 20mg/ml proteinase K (0.3mg/ml final)

Mix well by inverting tubes (DO NOT VORTEX)

Incubate at 37°C for 1hr (invert tubes several times to mix every 15min, DO NOT VORTEX)

Centrifuge (5000rpm, 5min, 4°C, Beckman JA-17 with adaptors)

Transfer supernatant (yellow and sticky) to 15ml conical tube.

Measure the volume (approx. 3ml) and adjust to 4.0 ml with TEN.

Add 4.2 g of cesium chloride and 10ul of Hoechst 33258 (10mg/ml)

Mix well by inverting tubes (DO NOT VORTEX)

Set up the gradient
Centrifuge tube : 1/2 x 2 in. (13 x 51 mm)
Label and tape (scotch) on side of the tubes
Gravity flow using syringe (3ml) and needle (18G ½) (Do not scratch tubes)
Fill tube with mineral oil (using syringe) up to a little above the shoulder of the tube
Seal the top by a heat-sealer
Set tubes in a rotor (vertical): pressure up to 60

Day2:
Start centrifugation (5h 30min at 80,000 rpm at 20°C (VTi90))

Unload DNA band (~1ml) to 14ml round bottom tube
Use 365nm UV light to protect DNA
Use 25G for an air vent, 18G to unload DNA
First, make an air vent with a 25G needle
Then, stick the 18G needle (with a 3ml syringe) a bit below the DNA band
Slowly unload DNA (~1ml)
Using the syringe graduation, measure the volume of DNA
Remove the needle when transferring DNA to a 14ml tube (to avoid shearing DNA)

Adjust the volume to 1.5ml with H_2O

Add 3.75 ml of 70% EtOH

Spin for 15 min at 8,000rpm (Beckman JA-17, 4˚C)

Discard supernatant

Suspend pellet in 1ml TE (Do not pipet. Just tap or invert tubes)

Add 10ul of 5M NaCl

Add 2.5ml of 100% EtOH

Spin for 15min at 8,000rpm (Beckman JA-17, 4˚C)

Wash pellet with 70% EtOH

Dry pellet (Air dry)

Suspend pellet in 150uL of TE (O/N at 4˚C, do not pipet)

Day3:
Measure DNA concentration both by agarose gel and Nano-Drop.
Transfer DNA to a 1.5ml tube

3. DNA digestion (example of *ori3001*)
Since *ori3001* is an origin in rDNA repeats, 1-5ug DNA is enough to detect replication intermediates. But for a single copy origin, 10ug should be digested.

KpnI-HindIII digest produce 3kb ori3001 fragment

DNA	2ug
10 x buffer #2 (NEB)	20uL
KpnI	3uL
HindIII	3uL
H2O to 200uL	

Incubate for 2hr at 37˚C
EtOH precipitation (20uL of 5M NaCl, 500uL of EtOH)
Centrifuge at 14 Krpm for 15min at RT
Suspend ppt in 15uL of loading buffer (30% glycerol, 10mM Tris-HCl pH8.0, 1mM EDTA pH8.0, bromophenol blue, xylene cyanol) (do not pipet, suspend pellet by scratching the wall and bottom of the tube by a tip

4. First dimension electrophoresis

First dimension gel preparation

[Gel Option 1]
0.4% agarose gel with 0.38ug/mL EtBr in 1 x TBE

260 mL of 1 x TBE
20uL of EtBr(5mg/mL)
1.04g agarose

[Gel Option 2]
0.4% agarose gel without EtBr in 1 x TBE

gel plate : 16 x 14.5 cm
comb : 20 lanes

gel box : distance of two poles : 26 cm
buffer : 1 x TBE without EtBr
20V constant voltage (0.04A)
run in dark place or cover gel box with aluminum foil
(if Gel Option 1 is used)
run gel o/n (depend on the desired fragment size)

- apply size marker DNA

Second dimension gel preparation

[Gel Option 1]; if Gel Option 1 is used for 1st dimension

buffer preparation
1 x TBE with 1ug/mL EtBr
3L of 1 x TBE + 0.6mL of EtBr(5mg/ml)
gel preparation
(without comb, the gel must be thicker than the 1st gel)
prepare 1% agarose with the buffer above.
300 mL of 1 x TBE with 1ug/mL EtBr
3g of agarose

[Gel Option 2]; if Gel Option 2 is used for 1st dimension

buffer preparation
1 x TBE with 0.33ug/mL EtBr
3L of 1 x TBE + 0.2mL of EtBr(5mg/ml)
gel preparation
(without comb, the gel must be thicker than the 1st gel)
prepare 1% agarose with the buffer above.
300 mL of 1 x TBE with 0.33ug/mL EtBr
3g of agarose
keep gel in cold room
gel plate : 21cm x 14.5 cm
gel box : distance of two poles : 26 cm

After second gel is solidified, cut trough away for first dimension gel slices.
Width of trough should be about 1cm.
The bottom edge of trough must be straight and smooth.
Save agarose gel removed during trough generation for next step

5. Gel slices for second dimension electrophoresis

After the first dimension gel electrophoresis, prepare gel slices for second dimension electrophoresis.
If Gel Option 2 is use, the 1st gel must be stained by 0.33ug/mL EtBr in TBE for 30min followed by de-staining in TBE.
Since KpnI-HindIII ars3001 fragment is 3kb, 1N position is 3kb and 2N position is 6kb.
In theory, 3kb-6kb gel slice is required.
But make 1.5kb-12kb gel slice just in case.
The distance between 1.5kb and 12kb should be about 3.5cm-4.5cm. (* in this way, 9 samples can be run in a gel)
Remove upper part (over 12 kb) and lower part (under 1.5kb) of gel
Make slice of each lane with sharp razor

5. Second dimension electrophoresis

Place gel slices in the trough.
Gel slices should be parallel with the bottom edge of the trough.
If gel slice attach to the bottom edge of the trough remove all bubbles between the 2 gels.
Melt the saved agarose and fill in around first dimension gel slices.
Run gel in cold room at 100V/0.12A for about 8 hr

6. Transfer to Hybond XL

After 2nd gel run, remove gel bumps and depurinate DNA by UV cross linker (UV stratalinker 1800)
*Optimal crosslink but stop at 400 not to over-depurinate

Transfer buffer : 0.4M NaOH, 1M NaCl
16g NaOH/L, 200ml of 5M NaCl
Transfer O/N (Transfer setting: See Southern Blotting Protocol, Use Hybond XL
UV link with UV stratalinker 1800
*Optimal crosslink but stop at 400 not to over-damage DNA
Wash membrane with 5 x SSC once or twice

7. hybridization

buffers

church buffer
(7% SDS, 1% BSA, 1mM EDTA pH8.0, 250mM NaPO4 pH7.2)

20% SDS	175mL
BSA	5g
0.5M EDTA	1mL
1M NaPO4 pH7.2	125mL
H2O to	500mL

Wash 1 (1L)

20 x SSC	100mL
20% SDS	5mL
H2O	895mL

Wash 2 (1L)

20 x SSC	10mL
20% SDS	10mL
H2O	980mL

Put the membrane into hybridization bottle containing 50ml of 5xSSC.
Discard 5xSCC
Add 20 mL of church buffer (hybridization buffer) to the bottle
Pre-hybridize membrane with Church buffer for 30min to O/N at 60C
Discard Church buffer and add fresh Church buffer (5mL for the short bottle, 10mL for the long bottle)

probe preparation

Boil 11uL of probe DNA for 5min
Chill on ice for 2min

Add 5uL of a-32P-dCTP (It depends on freshness and specificity of radioisotope)
Add 4uL of Highprime
Incubate 10min at 37C
Purify labeled DNA by G-25 column to remove unincorporated 32P (See its instruction)
Boil for 5min
Chill on ice

Add the probe to the hybridization bottle
Hybridize membrane at 60C for O/N

Rinse membrane with wash1 twice in the bottle
Wash membrane with wash1 at 50C for 10min twice in the bottle
Wash membrane with wash2 at 50C for 10min twice in the bottle

Dry membrane for 20min

Sandwich membrane in plastic wrap
Expose membrane to phosphoimager screen for 2 hr - O/N