Mitotic Spread

Solutions

Solution 1 (recipe for 100 ml)

- 8.02 ml K_2HPO_4 1 M
- 1.98 ml KH_2PO_4 1 M
- 21.864 g sorbitol (MWT 182.2)
- 50 ul $MgCl_2$ 1 M

Add 75 ml of water and dissolve, then pH to 7.4 with 3 M KOH and bring to 100 ml. Filter sterilize (do not autoclave).

Spheroblasting buffer (make fresh each time)

Solution 1 + 1/50th volume 1 M DTT + 1/50th volume zymolyase (from 10mg/ml 100T stock)

Solution 2 (recipe for 100 ml)

- 1.95 g MES
- 0.2 ml EDTA 0.5 M
- 50 ul $MgCl_2$ 1 M
- 18.22 g Sorbitol

Add 75 ml of water and dissolve, then pH to 6.4 with 3 M KOH and bring to 100 ml. Filter sterilize (do not autoclave).

20% Paraformaldehyde stock (make in fume hood)

1. Place 10 g paraformaldehyde in 50 ml conical tube.
2. Bring volume to 40 ml with water.
3. Add 0.5 ml 1 N NaOH and heat to 60º C in water bath.
4. When dissolved, bring to 50 ml with water and filter sterilize.
5. Store at room temperature for up to one year until use.

Fixative (make fresh each time in fume hood)

1. Mix 1 part of 20% paraformaldehyde stock with 4 parts of 4.25% sucrose stock (filter sterilize).
2. Every sample needs 120 ul of fixative overall = 24 ul of 20% paraformaldehyde + 96 ul of 4.25% sucrose.

Detergent

- 1% Kodak Photoflo (Hard to get — original protocol used 1% Lipsol.)

Filter sterilise and keep. Vary the concentration to increase the spreading.

Blocking buffer (make fresh each time)

- 0.1 g dried milk powder
- 0.25 g BSA
- 5 ml PBS

Procedure

Preparing the cells

1. Take 5 ODs of exponentially growing culture.
2. Spin down 1 min at 5000 rpm.
3. Resuspend in 1 ml (ice-cold) solution 1, transfer to Eppendorf tube and keep on ice. You can wait at this stage until the end of a time-course experiment.
4. Spin down 1 min at 6000 rpm in microcentrifuge.
5. Resuspend in 200 µl spheroblasting buffer.
6. Incubate 30 min at 37° C.

7. Check cell wall digestion. Mix a 1.5 µl sample (from the bottom of the Eppendorf) with 1.5 µl 2% SDS on a slide, add coverslip and check that >95% cells are lysed (usually there is a lot of cell debris — make sure you mix properly or it's difficult to tell). 30 min should be enough. Extend incubation if digestion is not complete.
8. Add 1 ml of ice-cold solution 2. Mix gently by inversion.
9. Spin down spheroplasts 8 min at 800 rpm. Aspirate supernatant carefully.
10. Gently resuspend in (ice-cold) solution 2 buffer. Keep on ice until spreading. (Samples can be left at this stage on ice overnight.)

Preparing the slides

1. Boil ~5 cm water in a large glass beaker in microwave.
2. Add 1/100 volume of 1M HCl (for 0.01 M).
3. Set up over Bunsen burner flame and stand slides up inside beaker (matt end up).
4. Bring to the boil (may have to cover top).
5. Boil for ~10 min.
6. Use forceps to take out slides and rinse them with 100% ethanol.
7. Lay out to dry — keep dust free!

Spreading (do in fume hood!)

1. Pipette 20 µl of gently resuspended spheroblasts onto centre of slide.
2. Prepare 3 full pipettes to carry out steps 3-5 as quickly as possible.
3. Add 40 µl fixative.
4. Add 80 µl detergent in a swirling motion.
5. Add 80 µl fixative is a swirling motion.
6. Use final pipette on its side to spread central area of slide without touching slide.
7. Remove bubbles.
8. Dry flat in fume hood. Original protocol says 2 h but always seems to need longer — do at least overnight, often over the weekend! Don't worry if they do not look dry after overnight drying as they have a 'wet-look'! Also, sometimes they look crystalline, which may seem worrisome, but there is not a noticable difference at the microscope stage.
9. Store spreads at −80° C. No problem with long term storage in slide boxes.

Immunostaining

1. Wash 10 min in PBS in Coplins jars.
2. Drain slide (for 2 s), add 200 µl blocking buffer to central area of slide.
3. Incubate 10 min at room temperature in humidity chamber.
4. Drain slide, add 200 µl 1° antibody in blocking buffer.
5. Add plastic square, incubate 1 h in humidity chamber.
6. Remove plastic square, wash twice ~10 min in PBS.
7. Repeat steps 4-6 for 2° antibody (incubate and wash in dark!).
8. Drain slide, add ~5 µl DAPI/antifade to centre of slide.
9. Add coverslip, cover slide with a tissue and press gently to spread DAPI and dry.
10. Paint edges of coverslip with nail varnish.