Indirect Immunofluorescence for Budding Yeast

Last updated 11/05/08 PBM.

Based on **CSH Yeast Genetics Course Handbook** and Kilmartin & Adams. (1984). *J Cell Biol*. 98(3), 922-933.

Keys to success:
- ***always preclear solutions by high speed microfuge spin just prior to use***
- ***might need to vary fixation time***
- ***might need to reduce formaldehyde concentration***

1. Grow cells to log phase. Up to O.D. 600 = 1.0 is okay.

2. Aliquot 5 mL or more of cells as needed to conical tube. Add 37% formaldehyde directly to culture (0.6 ml for every 5 mL of culture--final conc. is ~4%).

3. Put on 23°C roller drum, shaker or Nutator.to gently agitate cells during fixation.

4. Harvest fixed cells by centrifugation (e.g. Super T21 swinging bucket rotor—2 K for 3-5 minutes) after variable amount if time (if first time for a new protein, try 20-25 minutes as shortest and no more than 2 hours as longest incubation). For staining things like tubulin, 1-1.5 hours is good. *Note that fixation time influences time needed for spheroplasting*.

5. Wash cells 2-3 times with 5 mL **0.1 M $KHPO_4$, pH 6.5**.

6. Wash cells once with 5 mL **1.2 M sorbitol in 0.1 M $KPHO_4$** (aka K-sorb). Resuspend in 1.0 to 1.5 mL. At this point cells can be stored at 4°C for up to several days.

7. Usually I spheroplast 0.5 mL of cells and store rest at 4°C (just in case). To 0.5 mL of cells, add 0.5 mL of **1.2 M sorbitol in 0.1 M $KHPO_4$,** containing **5 µL ß-Mercaptoethanol**. Mix and let sit for a few minutes then add 15 µL **oxalyticase** (1 mg/mL stock). *Note one can also use Zymolyase 100T at 50 mg/ml final concentration or lyticase at ~50 units/ml final concentration.*

8. Incubate at 23°C on roller drum; check spheroplasting by phase optics after 20 min. Do not harvest cells until at least 50% of cells are phase dark and appear medium to dark gray in color. Short fixation samples with oxalyticase take around 20-25 minutes; longer fixation samples take around 40-45 min.

9. Harvest spheroplasts by gently spinning 2-3 min at ≤2 K. Wash once with 1.5 mL **1.2 M sorbitol in 0.1 M $KHPO_4$** using a P1000 to gently resuspend cells--NO VORTEXING. Resuspend washed cells in 0.5 mL **1.2 M sorbitol in 0.1 M $KHPO_4$**. Store spheroplasts on ice until ready to apply to microscope slides.

10. Apply 20 µL spheroplast suspension to each well of a **polylysine-coated microscope slide**. Let cells settle 10-20 min. in humidity chamber (e.g. a Nalgene tray with wet paper towels). Aspirate sups and immediately (but gently) plunge slide into **-20°C Methanol** for 6 min. *Note: For alternative protocol, see end.*

11. Transfer to **-20°C Acetone** for 30 seconds! *Note: Place Coplin jars containing organic solvents in a styrofoam box with a few pieces of dry ice to keep them cold.*

12. Allow slides to air-dry for 1-2 min. Put 20 μL **PBS-BSA** on each well. Put slide in humidity chamber, and incubate for at least 5 min. Blocking longer is usually better!

13. Aspirate **PBS-BSA** right before adding primary antibody (20 μL aliquots per well). Dilute primary antibody in **PBS-BSA**.

    Suggested dilutions
    1:1000-1:5000 for Boehringer 12CA5
    1:10,000 for VG43-2 (anti-tubulin)
    1:3,000 for C258-2 (anti-Smt3p)

14. Incubate at 4°C overnight. If you're in a hurry, several hours at R.T. might be sufficient.

15. Wash wells 4-5 times with **PBS-BSA**. Allow the later washes to sit for a few minutes.

16. Apply appropriate fluorescent secondary antibody (generally 1:500 to 1:2000). Incubate slides at R.T. in the dark for 2 hr.

17. Wash wells 4-5 times with **PBS-BSA**.

18. Wash 2 times with plain **PBS**.

19. Aspirate last wash and allow slides to air-dry in the dark.

20. Put a drop a mounting medium containing **DAPI** (~50 ng/mL) on each well. Put on cover slip, avoiding bubbles, and seal with nail polish. Store slides at -20°C.

ALTERNATIVE PERMEABILIZATION

After aspirating the PBS-BSA, just add a drop of **PBS-BSA plus 0.1% Tween 20** (PBST-BSA). Incubate for 15-20 min., then apply primary antibody diluted in PBST-BSA (or just PBS-BSA). DO NOT use any methanol or acetone.

REAGENTS NEEDED

Stock Solutions

1 M KH_2PO_4

1 M K_2HPO_4

2 M sorbitol

PBS ("CSH Recipe")

1x PBS	10 x PBS	2 Liters
0.04 M K_2HPO_4	0.4 M K_2HPO_4	139.3 g K_2HPO_4
0.01 M KH_2PO_4	0.1 M KH_2PO_4	27.2 g KH_2PO_4
0.15 M NaCl	1.5 M NaCl	175.3 g NaCl

Use stocks to make:

0.1 M $KHPO_4$, pH 6.5

1.2 M sorbitol in 0.1 M $KHPO_4$, pH 6.5

Note: For 0.1 M $KHPO_4$, pH 6.5, 0.328 mole fraction of total phosphate should be K_2HPO_4

e.g. For 100 ml 0.1 M KHPO4
3.28 ml 1 M K_2HPO_4
6.72 ml 1 M KH_2PO_4
90.0 ml H_2O

PBS-BSA (100 ml)

10x PBS	10 ml	(final 1x PBS)
BSA powder (Fraction V)	1 gram	(final 1% BSA)
10% Na Azide	1 ml	(final 0.1% NaN_3)

Adjust final volume to **100 ml**.
Best if let BSA dissolve at 4°C without stirring!
Store at 4°C.

Mounting Medium
Dissolve **50 mg p-phenylenediamine** (toxic) in **5 ml 1x PBS**.
Adjust to pH ~9.0 with NaOH (~50-60 μL of **1N NaOH**).
Check pH by spotting a few μl's onto pH paper.
Add 45 ml glycerol (autoclaved) and stir or mix until homogeneous (in airtight and dark container).
Store in aliquots in the -80°C freezer in airtight tubes in the dark.
If DNA-staining is also required, then add **DAPI to 50-100 ng/ml**.

Poly-lysine Coated Microscope Slides
Several samples can be processed on a multi-well Teflon-printed microscope slide (e.g. TEKDON, INC.). Prior to using slides, treat the wells with **0.1% polylysine** (>400,000 MW); prepared in water) for 10 min at RT. Rinse with distilled water and air-dry. Polylysine-coated slides can be prepared in advance and stored at R.T.; however, some people believe freshly prepared slides produce better results.