Gladfelter Lab Protocols

Single Molecule FISH and GFP booster in *Ashbya*

http://www.biosearchtech.com/steallarisprotocols

Modified from *S. cerevisiae* protocol

All steps must be performed in RNAse free conditions (see end for details)

Day 1

- [ ] Grow a 20 mL culture of *Ashbya* from dirty spores at 30°C for 15 hours. Add 2.2 mL 37% formaldehyde (final 3.7%) and shake at 30°C for 1 hour.

- [ ] Spin in 15 mL conicals at 300 rpm in tabletop centrifuge for 5 mins, discard supernatant in formaldehyde waste (in fume hood). Wash 2X with 5 mL ice cold Buffer B.

- [ ] Resuspend in 1 mL spheroplasting buffer, transfer to new microfuge tube.
    - If you have many cells, you may want to spin down and resuspend again in fresh spheroplasting buffer to make sure the cells are mostly in this buffer. Otherwise many of them may burst during digestion.

- [ ] Add 100 µL Zymolyase (15 mg/mL) and incubate on nutator at 37°C. Check digestion every 10 minutes until cells are phase-dark.
    - Wild-type cells take 40-50 minutes, but this varies by day and by strain.

- [ ] Spin at 2000 rpm (digested cells are very fragile) for 2 minutes in microfuge, and wash 2X with ice cold Buffer B.

- [ ] Spin a final time to remove Buffer B and resuspend in 1 mL 70% EtOH. Leave overnight at 4°C

Day 2

- [ ] Prepare hybridization solution: 100 µL hybridization buffer with 1-3 µL probe at the appropriate concentration. Vortex to mix thoroughly and centrifuge to get any droplets down into the bottom.
    - Warm the hybridization solution to room temperature before opening it.
    - For all the cyclin probes we use 1 µL.
    - For initial tests of probes, it is best to start 4 separate hybridization reactions by adding 1 µL each of the 1:10, 1:20, 1:50 and 1:100 working dilutions of the probes to see which is optimal.

- [ ] Centrifuge cells and remove the ethanol by pipetting.

- [ ] Resuspend in 1 mL of wash buffer. Let stand at room temperature 2-5 minutes.
    - Again if you have many cells, you may want to add a wash step to ensure you've removed as much ethanol as possible.

- [ ] Centrifuge sample and remove wash buffer. Add hybridization solution and incubate in the dark at 37°C overnight.

Day 3

- [ ] Add 1 mL wash buffer to the sample, invert to mix, centrifuge, and remove supernatant.
- [ ] Add a fresh 1 mL wash buffer and incubate at 37°C for 30 minutes.

IF NOT PERFORMING GFP BOOSTER

- [ ] Spin down cells, remove supernatant, resuspend in 500 µL wash buffer.
- [ ] Add 1 µL Hoechst and incubate in the dark at room temperature for 15 minutes.
- [ ] Wash with 500 µL wash buffer. Remove as much supernatant as possible.
- [ ] Add enough mounting medium to roughly equal the volume of cells. Use a minimum of 20 µL.
- [ ] Mount ~20 µL on glass slide and cover with long (24*50mm) coverslip. Press out bubbles and excess liquid and seal with nail polish. Slides may be stored at -20°C.

IF PERFORMING GFP BOOSTER

- [ ] Wash 2X with 250 µL 1X PBS + 1 mg/mL BSA. Resuspend a final time in 250 µL PBS + BSA, incubate in the dark at room temperature for 30 minutes to block.
- [ ] Wash 2X with 250 µL PBS + BSA.
- [ ] Resuspend in 200 µL PBS + BSA and add 1 µL GFP Booster and incubate overnight in the dark at 4°C.

Day 4

- [ ] Wash 2X with 250 µL PBS + BSA.
- [ ] Resuspend in 500 µL PBS + BSA. Add 1 µL Hoechst and incubate in the dark at room temperature for 15 minutes.
- [ ] Wash with 500 µL PBS + BSA. Remove as much supernatant as possible.
- [ ] Add enough Prolong Gold mounting medium to roughly equal the volume of cells. Use a minimum of 20 µL.
- [ ] Mount ~20 µL on glass slide and cover with long (24*50mm) coverslip. Press out bubble and excess liquid and seal with nail polish. Slides may be stored at -20°C.

RNAse free preparation:

Bottles, glass slides, and coverslips should be baked in the oven on the 3rd floor overnight to decontaminate.

Bottle tops should be liberally sprayed with RNAse Zap, covered with tin foil, and left overnight.

Triple-distilled water (Guerinot lab on the 3rd floor) is adequately RNAse free for our purposes and should be used to make solutions that will not be DPEC treated.

Each day bench, pipettes, tip boxes, and everything else sample may come in contact with shoud be wiped down with RNAse Zap wipes. Lab coat, gloves, and face mask should be worn at all times. It is a good idea to put up a sign at the bench reminding others to keep their distance during RNAse free work.

Solutions:

TE Buffer (50 mL):

78 mg	Tris-Cl (final 10 mM)
19 mg	EDTA (final 1 mM)

Dissolve Tris and EDTA in 30 mL H_2O. Adjust pH to 8.0 and make up to 50 mL. Treat with 50 µL DEPC. Incubate shaking at 30°C overnight, then autoclave for 15 minutes.

Probe Preparation:
Resuspend the oligonucleotide blend in 20 µL TE buffer (final concentration 250 µM).
Dilute this stock solution 1:10, 1:20, 1:50, and 1:100 in TE to make working dilutions. Test these to determine optimal concentration. (We use cyclins and Gpm2/3 at 1:10).
Store in the dark at -20°C.

Buffer B (100 mL):

60 mL	2 M Sorbitol
8.3 mL	1 M dibasic potassium phosphate
1.7 mL	1 M monobasic potassium phosphate
30 mL	H_2O

Treat with 0.1 mL DEPC per 100 mL. Incubate shaking at 30°C overnight, then autoclave for 15 minutes. Store at 4°C.

Spheroplasting buffer (10 mL):

10 mL	Buffer B
100 µL	200 mM Vanadyl ribonucleoside complex

Store at -20°C.

Zymolyase:
Make 15 mg/mL stock solution in RNAse free water. Store at -20°C.

70% Ethanol:
Make using RNAse free water.

20X SSC (500 mL):

87.65 g	NaCl
44.1 g	Na_3Citrate x 2 H_2O

Dissolve NaCl and Sodium Citrate in 400 mL H_2O. Adjust pH to 7.0 with a few drops of concentrated HCl. Adjust volume to 500 mL. Treat with 0.5 mL DEPC, incubate shaking at 30°C overnight, then autoclave for 15 minutes.

Hybridization Buffer (10 mL):

1 g	Dextran sulfate
10 mg	*E. coli* tRNA
100 µL	200 mM Vanadyl ribonucleoside complex
40 µL	50 mg/mL BSA (RNAse free)
1 mL	20X SSC
1 mL	Formamide (deionized)

Combine ingredients, make to 10 mL with RNAse free water. Store in 0.5 mL aliquots at -20°C.

1X PBS + BSA:
Make 1X PBS from 10X stock. DEPC treat (0.1 mL DEPC per 100 mL solution, incubate shaking at 30°C overnight, then autoclave for 15 minutes).

Make PBS + 1 mg/mL BSA fresh for each experiment using globulin-free lyophilized powder and store at 4°C.

Wash Buffer (50 mL):

5 mL	20X SSC
5 mL	Formamide (deionized)
40 mL	RNAse free H_2O

Hoechst & Prolong Gold Mounting Medium:
Use aliquots in common fridge, any RNAse seems to be diluted out enough during the incubation this isn't a problem. Do keep special "RNAse free only" aliquots, though.