



## FISH in Ashbya



sierrajc

**Abstract:** This is Fluorescent in situ Hybridization for looking at transcripts in Ashbya. If using this to look at RNA, then try to use all RNase-free material at the RNA bench. This was adapted from Therese Gerbich's protocol.

**Keywords:** Ashbya, Fluorescence, RNA, DNA

**Protocol Info:** sierrajc: FISH in Ashbya. [protocols.io](https://protocols.io/view/fish-in-ashbya-b4i6quhe)  
<https://protocols.io/view/fish-in-ashbya-b4i6quhe>

**Created:** Feb 01, 2022

**Last Modified:** Jun 13, 2022

**PROTOCOL integer ID:** 57662

### DEPC treated water

Add 100  $\mu$ l DEPC per 100 ml of ddH<sub>2</sub>O. Incubate shaking at 30 deg overnight, then autoclave for 15 mins

### TE Buffer

78 mg Tris-Cl (final concentration 10 mM)

19 mg EDTA (final concentration 1mM)

Dissolve Tris and EDTA in 30 mL H<sub>2</sub>O. Adjust pH to 8.0 and make up to 50 mL. Treat with 50  $\mu$ l DEPC. Incubate shaking at 30 deg overnight. Autoclave for 15 minutes.

### Buffer B (100 mL)

60 mL of 2M Sorbitol

8.3 mL 1M dibasic potassium phosphate

1.7 mL 1M monobasic potassium phosphate

30 mL H<sub>2</sub>O

Treat with 100  $\mu$ l DEPC. Incubate shaking at 30 deg overnight, then autoclave for 15 mins. Store at 4 deg (we keep ours in the cold room).

### Spheroplasting Buffer (10 mL)

10 mL Buffer B

100  $\mu$ l of 200 mM Vanadyl ribonucleoside complex

Make fresh each time.

70% RNase Free Ethanol

Make using RNase free water

RNase Free Zymolyase

Make 15mg/mL stock solution in RNase free water. Store at -20 deg.

20X SSC (500 mL)

87.65 g NaCl

44.1 g Sodium citrate

Dissolve NaCl and sodium citrate in 400 mL of H<sub>2</sub>O. Adjust pH to 7.0 with concentrated HCl. Adjust volume to 500 mL. Treat with 0.5 mL DEPC, incubate shaking at 30 deg overnight, then autoclave for 15 minutes

Hybridization Buffer

1 g Dextran Sulfate

10 mg E. coli tRNA

100  $\mu$ l 200 mM Vanadyl ribonucleoside complex

40  $\mu$ l 50 mg/mL BSA (RNase free)

1 mL 20X SSC

1 mL Formamide (deionized)

Combine ingredients, fill to 10 mL with RNase free water. Store in 0.5 mL aliquots in -20 deg (will be in Therese's stuff in a box labeled RNA FISH Buffers)

1X PBS + BSA

Make 1X PBS from 10X stock. DEPC treat (1 mL DEPC per 100 mL solution), incubate at 30 deg shaking overnight, and autoclave for 15 minutes.

With DEPC treated 1X PBS, add acetylated BSA lyophilized powder from the 4 deg fridge to make a 1mg/ml solution. This solution can be stored at 4 deg.

Wash Buffer (50mL)

5 mL 20X SSC

5 mL Formamide (deionized)

Fill to 50 mL with RNase free water.

Probe Preparation

Add 20  $\mu$ l of TE buffer to rehydrate the probe. This will create a 250  $\mu$ M solution of probe. Then make aliquots of proper dilution (1:10, 1:20, etc) depending on the probe.

### PMSF Preparation

Make a 10 mM stock solution with 17.42 mg PMSF in 10 mL RNase free EtOH. Store this in -20 deg for up to 4 months. When making dilution for use in protocol add 50  $\mu$ l PMSF stock to 500  $\mu$ l wash buffer.

### **Dry Materials**

Baffled Flasks

15 ml conical tubes

slides

coverslips

RNase free microcentrifuge tubes

RNase free pipette tips

RNase wipes

## Day 1

- 1 Grow 100mL culture of Ashbya from dirty spores, seeded at 2-3  $\mu$ L spores/mL culture at 30 deg for 15 hours in 1000 ml baffled flask. Start around 4.30 PM.

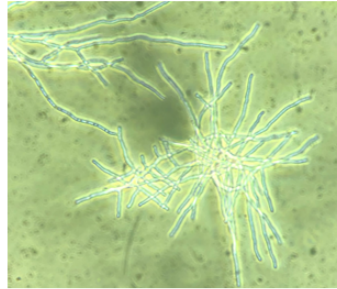
15h

## Day 2

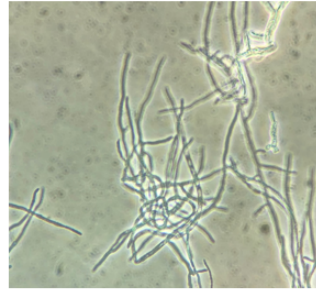
- 2 Add 10ml of 37% formaldehyde (final 3.7%) and shake for 1hr at 30 deg. Alternatively, first, spin cells down in 15 mL tubes, resuspend in 9 mL used media and add 1mL formaldehyde, to decrease PFA waste.
- 3 Spin in 15 ml conicals (Important! Do NOT use 50 mL tubes) at 400 rpm in tabletop centrifuge for 5 mins, discard supernatant in formaldehyde waste. Aspirate using 5 mL pipette vertically to avoid disturbing pellet.
- 4 Wash 2x with 5 mL ice-cold Buffer B. Make sure you have a visible white pellet at the end of this step. During this time make fresh spheroplasting buffer
- 5 Resuspend in 1 ml spheroplasting buffer and transfer to a new 1.5 ml microcentrifuge tube.

- 6** Add 100  $\mu\text{l}$  zymolyase (15 mg/ml) and incubate on a nutator at 37 deg. Check digestion every 5- 10 mins until cells phase dark. (*This varies by day and by strain. Do not over-digest or cells will lyse*)

#### Expected result



Not digested



Digested (dark phase)

- 7** Once phase dark, spin at 2000 rpm (digested cells are very fragile) for 2 mins in benchtop microfuge, wash 2x with 1 mL ice-cold buffer B. *At this stage, the cells will become hard to pellet. Aspirate around suspended clumps. Setting the tube in a stand for 2 minutes before aspirating media helps decrease cell loss. Don't worry about saving the hard-to-pellet cells, they are usually fragments of cells.*

#### **8 If doing proteinase k digestion**

- 8.1** Add a solution of 2.5  $\mu\text{l}$  Proteinase K in 500  $\mu\text{l}$  wash buffer to pellet and tap to mix.
- 8.2** Let cells sit in the proteinase k solution for 5 minutes at room temperature. *Don't let reaction proceed beyond this or cells will lyse.*
- 8.3** Spin at 2000 rpm for 2 mins and remove supernatant.

- 8.4** Add 500  $\mu\text{l}$  of 1mM PMSF in wash buffer to the cells, invert, and let it sit for 5 minutes. *This needs to be made fresh every time as PMSF is very unstable in aqueous solutions. Check that PMSF hasn't crashed out of solution in the stock.*
- 8.5** Wash once with wash buffer. Add 1 ml 70% RNase free EtOH leave overnight at 4 deg. Can be left in EtOH for a week.
- 9** Spin final time to remove Buffer B and resuspend in 1 ml 70% RNase free EtOH leave overnight at 4 deg. Cells should be in RNase free ethanol at 4 deg for at least 4 h. The cells may be stored in EtOH at 4 deg for up to a week.

### Day 3 or Evening of Day 2

- 10** Prepare hybridization solution: 100  $\mu\text{l}$  hybridization buffer with 1-3  $\mu\text{l}$  probe. Vortex to mix and centrifuge to remove any droplets.
- 10.1** Warm the hybridization solution to room temperature before opening
- 10.2** For initial tests of probes, it is best to start 4 separate hybridization reactions by adding 1  $\mu\text{l}$  each of the 1:10, 1:20, 1:50, 1:100 working dilutions to see which is optimal. *CLN3* and *BN1* seem to work well using 1-2  $\mu\text{l}$  of 25  $\mu\text{M}$  probe
- 11** Centrifuge cells at 2000 rpm for 2 mins and remove ethanol.
- 12** Resuspend in 1 ml of wash buffer let stand at room temperature 2-5 mins
- 13** Centrifuge samples and remove wash buffer. Add hybridization solution and incubate in the dark overnight at 37 deg on a nutator.

### Day 4 or Day 3

**14** Add 1 ml wash buffer to the sample, invert to mix, centrifuge (2000 rpm for 2 mins), and remove supernatant.

**15** Add 1 mL wash buffer and incubate at 37 deg for 30 min (to save time can add Hoechst to wash buffer)

**16 If not performing GFP booster**

**16.1** Spin down cells, remove supernatant, resuspend in 500  $\mu$ l wash buffer (can be skipped).

**16.2** Add 1 $\mu$ l Hoescht and incubate in the dark at room temperature for 15 min. (unless done previously in step 15)

**16.3** Wash with 500  $\mu$ l wash buffer. Remove as much supernatant as possible. *At this point, you may not even see a pellet. That's okay, as long as you had a small pellet till the previous step, you'll see cells later.*

**16.4** Add enough mounting medium to roughly equal the volume of cells. Use a minimum of 20  $\mu$ l. *Can increase if there are a lot of cells. Scrape the walls to get out the last of the cells, but make sure you don't make bubbles. Pipette up and down slowly to mix.*

**16.5** Mount 20  $\mu$ l on coverslip then place the slide on top. For coverslip use 22 $\times$ 50mm. (*This is important to not lose cells*). Gently and flatten with a notebook with something heavy on the slide to help flatten Ashbya.

**16.6** After about 10-15 mins of flattening, seal with nail polish. Slides may be stored at -20 deg.

**17 If performing GFP booster**

**17.1** Wash 2x with 250ul 1x-PBS + 1 mg/ml BSA. Resuspend a final time in 250  $\mu$ l PBS + BSA incubate in the dark at room temperature for 30 min to block.

- 17.2 Wash 2x with 250  $\mu$ l PBS+BSA
- 17.3 Resuspend in 200  $\mu$ l PBS +BSA and add 1  $\mu$ l GFP booster and incubate overnight in the dark at 4 deg
- 17.4 On the next day, wash 2X with 250  $\mu$ l PBS+BSA
- 17.5 Resuspend in 500  $\mu$ l PBS+BSA and 1ul Hoechst and incubate in the dark at room temperature for 30 min.
- 17.6 Wash with 500ul PBS + BSA, remove as much supernatant as possible.
- 17.7 Add enough mounting media to roughly equal the volume of the cells. Use a minimum of 20  $\mu$ l.
- 17.8 Mount 20  $\mu$ l on coverslip then place the slide on top. For coverslip use 22 $\times$ 50mm. (*This is important to not lose cells*). Gently and flatten with a notebook with something heavy on the slide to help flatten Ashbya.
- 17.9 After about 10-15 mins of flattening, seal with nail polish. Slides may be stored at -20 deg.