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Protocol status: Working
We use this protocol and it's working

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FISH in Ashbya

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ABSTRACT

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

MATERIALS

DEPC treated water
Add 100 µl DEPC per 100 ml of ddH2O. 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 H2O. Adjust pH to 8.0 and make up to 50 mL. Treat with 50 µl 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 H2O

Treat with 100 µl 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 µl 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 H2O. 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 µl 200 mM Vanadyl ribonucleoside complex
40 µl 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 mL20X SSC
5 mL Formamide (deionized)
Fill to 50 mL with RNAse free water.

Probe Preparation
Add 20 µl of TE buffer to rehydrate the probe. This will create a 250 µM 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 µ µl PMSF stock to500 µl 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 at2-3 µL 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.

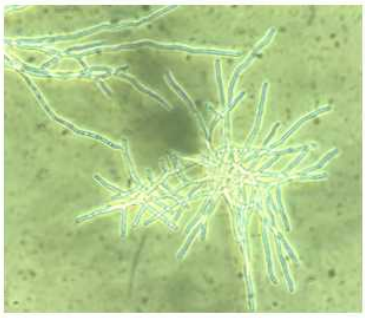
3 Spin in 15 ml conicals (Important! Do NOT use 50 mL tubes) at 400 rpm in tabletop centrifuge for 5 mins, discard supernatant in formaldehyde waste. Aspirate using 5 mL pipette vertically to avoid disturbing pellet.

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

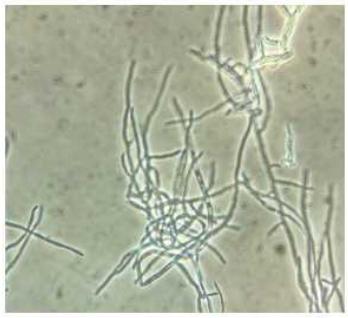
5 Resuspend in 1 ml spheroplasting buffer and transfer to a new 1.5 ml microcentrifuge tube.

6 Add 100 µl zymolyase (15 mg/ml) and incubate on a nutattor at 37 deg. Check digestion every 5-10 mins until cells phase dark. (T*his 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 µl Proteinase K in 500 µl 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 µl 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 µl hybridization buffer with 1-3 µl 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 µl each of the 1:10, 1:20, 1:50, 1:100 working dilutions to see which is optimal. *CLN3* and *BNI1* seem to work well using 1-2 µlof 25 µM 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 nutattor.

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 µl wash buffer (can be skipped).

16.2 Add 1µl Hoescht and incubate in the dark at room temperature for 15 min. (unless done previously in step 15)

16.3 Wash with 500 µl 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 µl. *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 µl on coverslip then place the slide on top. For coverslip use 22x50mm. (*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 l PBS + BSA incubate in the dark at room temperature for 30 min to block.

17.2 Wash 2x with 250 l PBS+BSA

17.3 Resuspend in 200 l PBS +BSA and add 1 l GFP booster and incubate overnight in the dark at 4 deg

17.4 On the next day, wash 2X with 250 l PBS+BSA

17.5 Resuspend in 500 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 µl.

17.8 Mount 20 µl on coverslip then place the slide on top. For coverslip use 22x50mm. (*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.