FISH Protocol for FISH & FLOW

Matthew Sullivan

Abstract

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Guidelines

Sample Collection

Biosphere 2 Samples

Materials
• Acid-Washed Glass Bottles (Enough for samples + 1 for collecting ocean water)
• 16% Formaldehyde
• 200 Proof Ethanol
• Ice & Cooler for transport (or 4°C equivalent)
• Serological Pipettes (25mL)
• Electronic Pipettor

Protocol
1. Submerge inverted capped acid-washed glass below the surface of the B2 ocean.
2. Remove cap while submerged and allow water to fill glass bottle. Swirl and empty.
3. Repeat Steps 1 & 2, but this time keep the seawater. This is your sample
4. Call B2 Energy Center to obtain B2 ocean temperature & salinity at time of sampling.
5. Add 70mL of 16% Formaldehyde to a new acid-washed glass bottle.
6. Add your sample to the bottle to q. to 1 liter.
7. Again repeat Steps 1 & 2 and keep the seawater.
8. Add 500mL of 200 Proof Ethanol to a new acid-washed glass bottle.
9. Add your sample to the bottle to q. to 1 liter.
10. Right before you leave the B2 Ocean, repeat Steps 1 & 2 and keep the seawater.
11. Add 1 liter of seawater to a new acid-washed glass.

Filtration Process

Materials
• 3-place filtration manifold (stainless steel or polyurethane)
• 3 glass microanalysis filter holder assemblies 47mm (tower, clamp, and base)
• 1 glass microanalysis filter holder assembly 90mm (tower, clamp, and base)
• 90mm GF/D Filter
• 47mm cellulose nitrate 0.45µm supor filter
• 47mm 0.2µm GTTP Isopore membrane filters

• 2 2 liter glass flasks with arm
• Tubing
• Vacuum pump

Protocol
1. Attach a 2 liter glass flask with arm to the vacuum pump using tubing.
2. Set up the 90mm filter holder assembly and add the 90mm GF/D filter.
3. Add your sample to the tower and turn on the vacuum to begin filtration. Make sure the vacuum pressure does not exceed 5 in Hg. The filtered product is called the pre-filtered product.
4. Set up the 47mm filter holder assembly with the flask and add the 0.2um GTTP Isopore membrane filters shiny-side up.
5. Filter the 15mL pre-filtered product per membrane filter (x3). Filter 100mL pre-filtered product per membrane filter (x9).
6. Use tweezers to remove membrane filters from the filter holder assembly and put on blotting paper in the dark shiny side up.
7. Once the membrane filter is dry, store in a petri dish.

CARD FISH
Reconstituting FISH probes

Materials
• Lyophilized Probe
• PCR water
• Ice & Ice Bucket
• Nanodrop System

Protocol
1. Add 100µL PCR water to lyophilized probe.
2. Incubate on ice for > 2 hours.
3. Finger-flick a few times and shake down material to bottom of tube. (NOTE: you do not want to vortex because HRP enzyme attached to the probe may detach)
4. Use 1.5µL of reconstituted probe and check the UV at 260 and 404 wavelengths on the Nanodrop.
5. Add PCR water depending on Nanodrop results.

Embedding Samples
***NOTE: For microscopy samples only!

Materials
• Low Gelling Point Agarose
• Parafilm
• Glass Slide
• 80-96% Ethanol
• Tweezers (Non-fine)
• 2 Large-sized Petri Dishes
• Sample filtered on 47mm 0.2µm GTTP Isopore membrane filters

Protocol
1. Pre-warm a petri-dish.
2. Boil 0.1% low gelling point agarose and pour into the pre-warmed petri-dish.

3. Let agarose cool down to 35-40°C.
4. Cover glass slides with layer of parafilm so that there is an even surface.
5. Using sterile tweezers, dip filter with both sides in the agarose and place it face-down (ie. shiny-side/bacteria-side down!) onto the parafilm-covered slide.
6. Let dry at room temperature.
7. Remove filter from slide surface by soaking parafilm covered slide in petri-dish filled with 80-96% Ethanol.
8. Air-dry filter on a kimwipe.

Pre-Treatment

Materials
• Lysozyme Solution (10mg/mL)
• Recipe (10mL):
  • 1mL 0.5M EDTA pH 8.0
  • 3mL 1M Tris-HCl pH 8.0
  • 6mL MQ water (autoclaved)
  • 100mg Lysozyme (Fisher Cat#BP535-5)
• 0.01M HCl
• Water Bath
• Tweezers (Non-fine)
• Sterile Scalpel
• Blotting paper
• Pencil
• 2 Medium-sized Petri Dishes
• Sample filtered on 47mm 0.2µm GTTP Isopore membrane filters

Protocol
1. Turn on water bath and pre-warm to 37°C and sterilize tweezers with 70% EtOH.
2. While the water bath is warming up, take out a piece of blotting paper. Put your sample filter shiny-side up on the blotting paper. Cut your sample filters in half using sterile scalpel and sterile tweezers to stabilize. Mark the filter piece using a pencil.
3. Make fresh lysozyme solution and pour it into a petri dish.
4. To *permeabilize* your sample, submerge your samples in the lysozyme solution. Wrap parafilm around the edge of the petri-dish.
5. Put the petri-dish into the 37°C water bath for 1 hour.
6. While waiting for your incubation, pour 0.01M HCl in a new petri-dish.
7. Once your incubation is over, put your sample filters in a strainer and rinse off any residual lysozyme solution with distilled water.
8. To *inactivate of endogenous peroxidases*, submerge your sample in the 0.01M HCl and incubate for 15 minutes.
9. Again, put your sample filters in a strainer and rinse off any residual lysozyme solution with distilled water.
10. Air dry on a kimwipe.

Hybridization

Materials
• Probe

- Hybridization Chamber Mix
  - Recipe (2mL)
    - % Formamide
    - q. to 2mL with MQ water
- Hybridization Buffer
  - Recipe (refer to Elke's recipe)
- Hybridization Ovens
- Tweezers (Non-fine)
- Sterile Scalpel
- Blotting paper
- Pencil
- Sample filtered on 47mm 0.2µm GTTP Isopore membrane filters
- 50mL falcon tubes
- Styrofoam 50mL falcon tube holder
- Cardboard
- Glass slides
- Kimwipes
- Parafilm

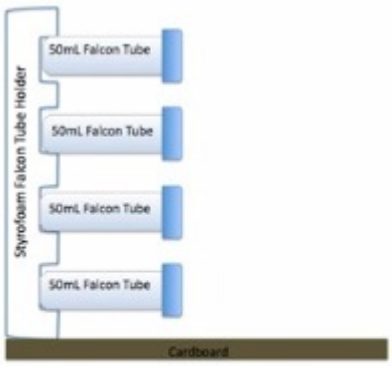

Fig. 1. Hybridization Humidity Chamber Setup

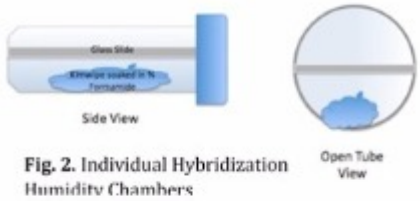

Fig. 2. Individual Hybridization Humidity Chambers

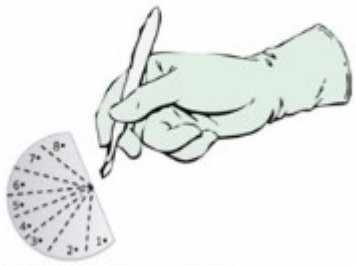

Fig. 3. Cutting the sample filter

Protocol

1. Pre-heat hybridization ovens to 46°C and 48°C.
2. Create a hybridization humidity chamber setup (refer to *Fig. 1*).

3. Based on the probe you plan to use for hybridization, determine the % formamide to use.
4. Create the individual hybridization humidity chambers (*Fig. 2*) by inserting a kimwipe soaked in the correct **2mL % formamide-water mix (Hybridization Chamber Mix)**. Cover glass slides evenly with parafilm. Do NOT add slide to individual chamber yet.
a. eg. If probe needs 35% formamide, create hybridization chamber mix that is 35% formamide (700µL formamide, 1300µL MQ water)
5. Take out a piece of blotting paper. Put your sample filter shiny-side up on the blotting paper. Cut your sample already halved filters into eighths using sterile scalpel and sterile tweezers to stabilize. Number the filter pieces using a pencil with a "#" followed by "•".
a. NOTE: The "•" indicates the orientation of the filter, once the "shine" is no longer obvious
6. Mix **hybridization buffer** with **probe working solution** [50ng DNA μl^{-1}] in a 300:1 ratio.
7. Dip each filter completely into the **Hybridization mix** and place filters face-up on the parafilm covered glass slide. Spread the rest of the solution evenly onto the filters.
8. Put glass slide horizontally into individual hybridization humidity chamber with corresponding % formamide to probe.
9. Incubate at 46°C overnight.

Washing
*** Prepare wash buffer at same time as setting up hybridization

Materials
• Washing Buffer
  • Recipe (refer to Elke's recipe)
• 1x PBS
• H_2O_2
• Amplification Buffer
  • Recipe (refer to Elke's recipe)
• Alexa 488
• EtOH
• Ice
• Tweezers (Non-fine)
• Large-sized Petri Dishes
• 50mL Falcon Tubes
• Glass slides
• Kimwipes
• Parafilm

Protocol
Prior Day
1. Make 50mL Washing Buffer in 50mL falcon tube with corresponding % formamide to probe.
2. Warm at 48°C overnight.
Day Of
3. Cover glass slides evenly with parafilm. Set aside until later use.
4. Remove filters from individual humidity chambers and put in corresponding % formamide pre-warmed **Washing Buffer**. Incubate at 48°C for 10 minutes.
5. Pour 1x PBS in a Large-sized petri dish.
6. Transfer filters to 1x PBS and incubate for 15 minutes at room temperature.
7. Get **Ice** and put H_2O_2, Amplification Buffer, Alexa 488 in covered ice bucket.
8. Mix 1µL H_2O_2 with 199µL 1x PBS.

9. Create the **Substrate mix** in a 1000 **Amplification Buffer**: 10 **diluted H_2O_2** : 3.3 **Alexa 488** ratio. Keep in covered ice bucket until ready to use.
a. Eg. Substrate Mix: 1000µL Amplification Buffer, 10µL diluted H2O2, 3.3µL Alexa 488.
10. Take filters out of 1x PBS and quickly wipe off excess 1x PBS on kimwipe. Make sure NOT to touch to cell-side to the kimwipe.
11. Dip filters into the **Substrate mix** and put on parafilm covered slide. Spread the rest of the mix evenly onto the filters.
12. Put slides into large-sized petri dishes and seal petri dish with parafilm and put in 46°C for 45 minutes in the dark.
13. Dry filters on kimwipe and put in 1x PBS for 10 minutes at room temperature in the dark.
14. Pour MQ water into a Large-sized petri dish and pour 96% EtOH into a different Large-sized petri dish.
15. Transfer filters to MQ water and cover in dark for 1 min at room temperature.
16. Transfer filters to 96% EtOH and cover in dark for 1 min at room temperature.
17. Put on kimwipe on a paper towel and let dry covered in the dark.
18. Immediately begin resuspension step! NOTE: If for microscopy, you can store filters at -20°C.

Resuspension of Cells from Filters

Materials
• 150mM NaCl
• 10% Tween 80 Ampules
• 0.2µm syringe filter
• Luer-lok syringe
• Large-sized petri dish
• 2mL centrifuge tubes
• Tape
• Incubator
• Vortexers

Protocol
1. Pre-heat incubator to 37°C.
2. Pour 1x PBS in a Large-sized petri dish. Transfer hybridized filters to 1x PBS and incubate for 15 minutes in the dark at room temperature.
3. 0.2µm filter sterilize the 150mM NaCl.
4. Make **Resuspension Buffer** (30mL 150mM NaCl; 160µL 10% Tween 80)
5. Put 1.5mL Resuspension Buffer per 2mL centrifuge tube and put filter in centrifuge tube.
6. Incubate centrifuge tube for 30 minutes at 37°C shaking horizontally.
7. Tape Tubes to vortexer horizontally (6 tubes per for vortexer). Shake in dark for 15 minutes at 2500 rpm.
8. Remove filter (BUT keep filter just in case). The cells should now be in the buffer!!

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Before start

Prepare wash buffer at same time as setting up hybridization.

Protocol

Sample Collection

Step 1.
Submerge inverted capped acid-washed glass below the surface of the B2 ocean.

Sample Collection

Step 2.
Remove cap while submerged and allow water to fill glass bottle.

Sample Collection

Step 3.
Swirl and empty.

Sample Collection

Step 4.
Repeat Steps 1-3, but this time keep the seawater.

NOTES
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This is your sample.

Sample Collection

Step 5.
Call B2 Energy Center to obtain B2 ocean temperature & salinity at time of sampling.

Sample Collection

Step 6.
Add 70mL of 16% Formaldehyde to a new acid-washed glass bottle.

Sample Collection

Step 7.
Add your sample to the bottle to q. to 1 liter.

Sample Collection

Step 8.
Again repeat Steps 1-3 and keep the seawater.

Sample Collection

Step 9.
Add 500mL of 200 Proof Ethanol to a new acid-washed glass bottle.

Sample Collection

Step 10.
Add your sample to the bottle to q. to 1 liter.

Sample Collection

Step 11.
Right before you leave the B2 Ocean, repeat Steps 1-3 and keep the seawater.

Sample Collection

Step 12.
Add 1 liter of seawater to a new acid-washed glass.

Filtration Process

Step 13.
Attach a 2 liter glass flask with arm to the vacuum pump using tubing.

Filtration Process

Step 14.
Set up the 90mm filter holder assembly and add the 90mm GF/D filter.

Filtration Process

Step 15.
Add your sample to the tower and turn on the vacuum to begin filtration.

NOTES

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The filtered product is called the pre-filtered product.

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Make sure the vacuum pressure does not exceed 5 in Hg.

VERVE Team 21 Jan 2016
Make sure the vacuum pressure does not exceed 5 in Hg.

Filtration Process

Step 16.
Set up the 47mm filter holder assembly with the flask and add the 0.2um GTTP Isopore membrane filters shiny-side up.

Filtration Process

Step 17.
Filter the 15mL pre-filtered product per membrane filter (x3).

Filtration Process

Step 18.
Filter 100mL pre-filtered product per membrane filter (x9).

Filtration Process

Step 19.
Use tweezers to remove membrane filters from the filter holder assembly and put on blotting paper in the dark shiny side up.

Filtration Process

Step 20.
Once the membrane filter is dry, store in a petri dish.

Reconstituting FISH probes

Step 21.
Add 100µL PCR water to lyophilized probe.

Reconstituting FISH probes

Step 22.
Incubate on ice for > 2 hours.

Reconstituting FISH probes

Step 23.
Finger-flick a few times and shake down material to bottom of tube.

NOTES
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NOTE: you do not want to vortex because HRP enzyme attached to the probe may detach.

Reconstituting FISH probes

Step 24.
Use 1.5µL of reconstituted probe and check the UV at 260 and 404 wavelengths on the Nanodrop.

Reconstituting FISH probes

Step 25.
Add PCR water depending on Nanodrop results.

Embedding Samples (For microscopy samples only)

Step 26.
Pre-warm a petri-dish.

Embedding Samples (For microscopy samples only)

Step 27.
Boil 0.1% low gelling point agarose and pour into the pre-warmed petri-dish.

Embedding Samples (For microscopy samples only)

Step 28.
Let agarose cool down to 35-40°C.

Embedding Samples (For microscopy samples only)

Step 29.
Cover glass slides with layer of parafilm so that there is an even surface.

Embedding Samples (For microscopy samples only)

Step 30.
Using sterile tweezers, dip filter with both sides in the agarose and place it face-down (ie. shiny-side/bacteria-side down!) onto the parafilm-covered slide.

Embedding Samples (For microscopy samples only)

Step 31.

Let dry at room temperature.

Embedding Samples (For microscopy samples only)

Step 32.
Remove filter from slide surface by soaking parafilm covered slide in petri-dish filled with 80-96% Ethanol.

Embedding Samples (For microscopy samples only)

Step 33.
Air-dry filter on a kimwipe.

Pre-Treatment

Step 34.
Turn on water bath and pre-warm to 37°C and sterilize tweezers with 70% EtOH.

Pre-Treatment

Step 35.

While the water bath is warming up, take out a piece of blotting paper.

Pre-Treatment

Step 36.

Put your sample filter shiny-side up on the blotting paper.

Pre-Treatment

Step 37.

Cut your sample filters in half using sterile scalpel and sterile tweezers to stabilize.

Pre-Treatment

Step 38.

Mark the filter piece using a pencil.

Pre-Treatment

Step 39.

Make fresh lysozyme solution and pour it into a petri dish.

Pre-Treatment

Step 40.

To *permeabilize* your sample, submerge your samples in the lysozyme solution.

Pre-Treatment

Step 41.

Wrap parafilm around the edge of the petri-dish.

Pre-Treatment

Step 42.

Put the petri-dish into the 37°C water bath for 1 hour.

DURATION

01:00:00

Pre-Treatment

Step 43.

While waiting for your incubation, pour 0.01M HCl in a new petri-dish.

Pre-Treatment

Step 44.

Once your incubation is over, put your sample filters in a strainer and rinse off any residual lysozyme solution with distilled water.

Pre-Treatment

Step 45.

To *inactivate of endogenous peroxidases*, submerge your sample in the 0.01M HCl and incubate for 15 minutes.

DURATION

00:15:00

Pre-Treatment

Step 46.

Again, put your sample filters in a strainer and rinse off any residual lysozyme solution with distilled water.

Pre-Treatment

Step 47.

Air dry on a kimwipe.

Hybridization

Step 48.
Pre-heat hybridization ovens to 46°C and 48°C.

Hybridization

Step 49.
Create a hybridization humidity chamber setup.

NOTES
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Refer to *Fig. 1* in guidelines.

Hybridization

Step 50.
Based on the probe you plan to use for hybridization, determine the % formamide to use.

Hybridization

Step 51.
Create the individual hybridization humidity chambers (*Fig. 2* in guidelines) by inserting a kimwipe soaked in the correct **2mL % formamide-water mix (Hybridization Chamber Mix)**.

NOTES
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eg. If probe needs 35% formamide, create hybridization chamber mix that is 35% formamide (700µL formamide, 1300µL MQ water).

Hybridization

Step 52.
Cover glass slides evenly with parafilm. Do NOT add slide to individual chamber yet.

Hybridization

Step 53.
Take out a piece of blotting paper.

Hybridization

Step 54.
Put your sample filter shiny-side up on the blotting paper.

Hybridization

Step 55.
Cut your sample already halved filters into eighths using sterile scalpel and sterile tweezers to stabilize.

Hybridization

Step 56.
Number the filter pieces using a pencil with a "#" followed by "•".

NOTES
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NOTE: The "•" indicates the orientation of the filter, once the "shine" is no longer obvious.

Hybridization

Step 57.
Mix **hybridization buffer** with **probe working solution** [50ng DNA μl^{-1}] in a 300:1 ratio.

Hybridization

Step 58.
Dip each filter completely into the **Hybridization mix** and place filters face-up on the parafilm covered glass slide.

Hybridization

Step 59.
Spread the rest of the solution evenly onto the filters.

Hybridization

Step 60.
Put glass slide horizontally into individual hybridization humidity chamber with corresponding % formamide to probe.

Hybridization

Step 61.
Incubate at 46°C overnight.

DURATION
18:00:00

Washing: Prior Day

Step 62.
Make 50mL Washing Buffer in 50mL falcon tube with corresponding % formamide to probe.

Washing: Prior Day

Step 63.
Warm at 48°C overnight.

DURATION
18:00:00

Washing: Day Of

Step 64.
Cover glass slides evenly with parafilm. Set aside until later use.

Washing: Day Of

Step 65.
Remove filters from individual humidity chambers and put in corresponding % formamide pre-warmed **Washing Buffer**.

Washing: Day Of

Step 66.
Incubate at 48°C for 10 minutes.

DURATION
00:10:00

Washing: Day Of

Step 67.
Pour 1x PBS in a Large-sized petri dish.

Washing: Day Of

Step 68.
Transfer filters to 1x PBS and incubate for 15 minutes at room temperature.

DURATION
00:15:00

Washing: Day Of

Step 69.
Get Ice and put H_2O_2, Amplification Buffer, Alexa 488 in covered ice bucket.

Washing: Day Of

Step 70.

Mix 1µL H_2O_2 with 199µL 1x PBS.

Washing: Day Of

Step 71.

Create the Substrate mix in a 1000 Amplification Buffer: 10 diluted H_2O_2 : 3.3 Alexa 488 ratio.

NOTES

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Eg. Substrate Mix: 1000µL Amplification Buffer, 10µL diluted H_2O_2, 3.3µL Alexa 488.

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Keep in covered ice bucket until ready to use.

Washing: Day Of

Step 72.

Take filters out of 1x PBS and quickly wipe off excess 1x PBS on kimwipe.

NOTES

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Make sure NOT to touch to cell-side to the kimwipe.

Washing: Day Of

Step 73.

Dip filters into the Substrate mix and put on parafilm covered slide.

Washing: Day Of

Step 74.

Spread the rest of the mix evenly onto the filters.

Washing: Day Of

Step 75.

Put slides into large-sized petri dishes and seal petri dish with parafilm and put in 46°C for 45 minutes in the dark.

DURATION

00:45:00

Washing: Day Of

Step 76.

Dry filters on kimwipe and put in 1x PBS for 10 minutes at room temperature in the dark.

DURATION

00:10:00

Washing: Day Of

Step 77.

Pour MQ water into a Large-sized petri dish and pour 96% EtOH into a different Large-sized petri dish.

Washing: Day Of

Step 78.

Transfer filters to MQ water and cover in dark for 1 min at room temperature.

DURATION

00:01:00

Washing: Day Of

Step 79.

Transfer filters to 96% EtOH and cover in dark for 1 min at room temperature.

DURATION
00:01:00

Washing: Day Of

Step 80.
Put on kimwipe on a paper towel and let dry covered in the dark.

Washing: Day Of

Step 81.
Immediately begin resuspension step!

NOTES
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NOTE: If for microscopy, you can store filters at -20°C.

Resuspension of Cells from Filters

Step 82.
Pre-heat incubator to 37°C.

Resuspension of Cells from Filters

Step 83.
Pour 1x PBS in a Large-sized petri dish.

Resuspension of Cells from Filters

Step 84.
Transfer hybridized filters to 1x PBS and incubate for 15 minutes in the dark at room temperature.

DURATION
00:15:00

Resuspension of Cells from Filters

Step 85.
0.2µm filter sterilize the 150mM NaCl.

Resuspension of Cells from Filters

Step 86.
Make **Resuspension Buffer** (30mL 150mM NaCl; 160µL 10% Tween 80).

Resuspension of Cells from Filters

Step 87.
Put 1.5mL Resuspension Buffer per 2mL centrifuge tube and put filter in centrifuge tube.

Resuspension of Cells from Filters

Step 88.
Incubate centrifuge tube for 30 minutes at 37°C shaking horizontally.

DURATION
00:30:00

Resuspension of Cells from Filters

Step 89.
Tape Tubes to vortexer horizontally (6 tubes per for vortexer).

Resuspension of Cells from Filters

Step 90.
Shake in dark for 15 minutes at 2500 rpm.

DURATION
00:15:00

Resuspension of Cells from Filters

Step 91.
Remove filter (BUT keep filter just in case).

NOTES
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The cells should now be in the buffer!!