

### C. CARD FISH

In environmental samples, single oligonucleotides carrying only one fluorochrome may not provide enough fluorescence signal to detect cells with **low ribosome content** (Pernthaler et al., 2002). **Polynucleotide** probes with a length of more than 100 nucleotides carrying several fluorochromes are an alternative (Trebesius et al., 1994; DeLong et al., 1999). However, these probes **lack specificity** for narrow target groups which are at the level of species and genera. An alternative labeling technique that increases fluorescence signals intensity uses **horseradish-peroxidase (HRP-) labeled oligonucleotides**. When using HRP-labeled probes, fluorescent staining results from a **secondary incubation with fluorescently labeled tyramide**. The specifically bound peroxidase molecules catalyze the deposition of these labeled reporter compounds within cells targeted by the HRP tagged probe. FISH signals are up to **20-fold brighter** with HRP labeled probes than with conventional single labeled probes (Schönhuber et al., 1997). However, cell permeabilization protocols need to be adjusted in order to enable the larger enzyme-labeled oligonucleotides to penetrate into the cells (Pernthaler et al., 2002).

Depending on the sample the protocols for CARD-FISH differ substantially. Generally there are two additional steps in the protocols, embedding in agarose and tyramide incubation. The former is necessary to prevent cell loss due to the permeabilisation. It is also thought to have a cell stabilising effect. Tyramide incubation is needed for mediating the signal amplification process.

Here we present protocols for (1) plankton, (2) sediment, and (3) tissues.

**NOTE: Please refer to page 8 (last page) for detailed information on (4) the hybridization, washing and CARD amplification buffers. They apply to all three protocols.**

#### Literature:

Pernthaler, A., J. Pernthaler, and R. Amann. 2002. *Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. Applied and Environmental Microbiology* 68:3094-3101

Trebesius, K., R. Amann, W. Ludwig, K. Mühlegger, and K.-H. Schleifer. 1994. *Identification of whole fixed bacterial cells with nonradioactive 23S rRNA-targeted polynucleotide probes. Applied and Environmental Microbiology* 60:3228-3235.

DeLong, E., L. Taylor, T. Marsh, and C. Preston. 1999. *Visualization and enumeration of marine planktonic archaea and bacteria by using polyribonucleotide probes and fluorescent in situ hybridization. Applied and Environmental Microbiology* 65:5554-5563.

Schönhuber, W., B. Fuchs, S. Juretschko, and R. Amann. 1997. *Improved sensitivity of whole-cell hybridization by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. Applied and Environmental Microbiology* 63:3268-3273.

## 1. CARD-FISH protocol for planktonic samples on membrane filters (according to Pernthaler et al., 2004)

**please cite for this protocol:** Pernthaler, A., J. Pernthaler, and R. Amann. 2004. Sensitive multi-color fluorescence in situ hybridization for the identification of environmental microorganisms, p. 711-726. In G. Kowalchuk, F. J. de Bruijn, I. M. Head, A. D. L. Akkermans, and J. D. van Elsas (ed.), *Molecular Microbial Ecology Manual*, 2nd 3.11 ed. Kluwer Academic Publishers, Dordrecht, Boston, London.

### Embedding

1. Boil low gelling point agarose (0.1%, gel strength should be approx. 1000 g cm<sup>-2</sup>).
2. Fill the agarose in a petri dish and let it cool down to 35-40 °C.
3. Dip filter with both sides in the agarose and place it face-down onto a parafilm covered, even surface (e.g. glass plate), let dry; Temperature for drying is not crucial, anything between 20 – 50 °C is fine.
4. Remove filters from surface by soaking in 80 – 96% ethanol (Use a lot of ethanol for removal of the filter, if the filter sticks to the parafilm - use more ethanol)
5. Let the filter air dry on Kimwipes/ Whatman paper\*

### Permeabilization

(Permeabilization with lysozyme proved to be the optimal method for most of the marine planktonic bacteria)

1. Incubate filter in fresh lysozyme solution [10 mg ml<sup>-1</sup> in 0.05M EDTA, pH 8.0; 0.1M Tris-HCl, pH 8.0] for 60 min at 37 °C
2. Wash in excess MilliQ water

(No ethanol washing is necessary at this point; proceed immediately to the inactivation of endogenous peroxidases.)

### Inactivation of endogenous peroxidases

1. Incubate in 0.01 M HCl for 10 - 20 min at RT
2. Wash filters well in excess MilliQ water
3. Wash 96% ethanol and let the filter air dry on Kimwipes/ Whatman paper\*.

If you have problems with autofluorescence of your cells, an additional incubation in 3% H<sub>2</sub>O<sub>2</sub> in water for 10 min @ RT may help.

### Hybridization in humidity chamber

Prepare a humidity chamber by inserting a tissue in a 50 ml tube and soak it with a formamide-water mix according to the formamide concentration of the hybridization buffer

1. Mix hybridization buffer with probe working solution [50 ng DNA µl<sup>-1</sup>] in a ratio 300:1;
2. Dip each filter completely into the hybridization solution and place filters face-up onto a parafilm covered glass slide; spread the rest of the solution evenly onto the filters;

- close tube firmly and keep the tube in a horizontal position (e.g. in an appropriate rack)
3. Incubate at 46°C for 2-3 h (coastal water) or overnight for oligotrophic/open ocean water samples

## Washing

Transfer filter to the washing buffer by immersing the whole slide in the prewarmed washing buffer => filter will swim off

1. Wash filters in prewarmed washing buffer (10 min, 48°C)
2. Transfer filters to 1 x PBS (do not let filter run dry!) and incubate for 15 min @ RT
3. To remove excess liquid, dab filter on blotting paper, but **do not let filter run dry**

## CARD

1. Prepare a fresh solution of H<sub>2</sub>O<sub>2</sub> (0.15% in PBS), keep it cool
2. Mix amplification buffer with H<sub>2</sub>O<sub>2</sub> solution in a ratio of 100:1 (as a guideline: the same volume as the hybridization mix is sufficient)
3. Add fluorescently labeled tyramide [1 mg dye ml<sup>-1</sup>] and mix well, keep in the dark

(The volume of labeled tyramide added strongly depends on the nature of the sample, start with 1:1000; if the signal is not sufficient: in-/decrease the ratio of added tyramide.)

4. Dip filter completely in the amplification mix, place filter sections face-up on a parafilm covered glass slide and put this e.g. into a petri dish; spread the rest of the amplification mix over the filters and incubate at 46°C for up to 45 min in the dark!
5. To remove excess liquid, dab filter on blotting paper and incubate in 1 x PBS for 5-10 min at RT in the dark
6. Wash filters thoroughly in excess MQ and twice in 96% ethanol (~1 min), let completely air dry in the dark before counterstaining with DAPI
7. To keep the background fluorescence low, it is important to wash the filters in large volumes of water and ethanol

\* Filters may now be stored in the freezer without apparent loss in CARD-FISH signal intensity. RT: room temperature

## 2. CARD-FISH protocol for sediment samples on membrane filters (according to Ishii et al., 2004)

***please cite for this protocol:*** Ishii, K., M. Mußmann, B. J. MacGregor, and R. Amann. 2004. An improved fluorescence in situ hybridization protocol for the identification of bacteria and archaea in marine sediments. *FEMS Microbiology Ecology* 50:203-212.

### Sonication and filtration

(Sonication protocol depends on sediment type and sampling depth, for some sandy sediments the following procedure turned out to be successful)

1. Put fixed sediment sample on ice and sonicate it 7 times for 30 sec with a 30 sec break in between with 1 pulse per second and an intensity of 20% (Sonopuls HD 70, Bandelin, Berlin, Germany)
2. Vortex sample and filtrate suitable amounts of the supernatant onto polycarbonate filter (pressure: -200 to 400 mbar)
3. Wash twice with sterile MQ or 1 x PBS pH 7.6
4. Let air dry \*

### Embedding

1. Boil low gelling point agarose [0.1%, gel strength should be approx. 1000 g cm<sup>-2</sup>] and let it cool down to 35-40°C.
2. To avoid cross-contamination dip each filter with both sides in a separate portion of agarose and place it face-down onto a parafilm covered, even surface (e.g. glass plate), let dry at 20 – 50°C
3. Remove filters from surface by soaking in 80 – 96% ethanol

(Use a lot of ethanol for removal of the filter, if the filter sticks to the parafilm - use more ethanol)

4. Let the filter air dry on Kimwipes/ Whatman paper\*

### Permeabilization

(Permeabilization with lysozyme proved to be the optimal method for most of the marine planktonic bacteria)

1. Incubate filter in fresh lysozyme solution [10 mg ml<sup>-1</sup> in 0.05 M EDTA, pH 8.0; 0.1M Tris-HCl, pH 8.0] for 60 min at 37°C
2. Wash in excess MilliQ water

(No ethanol washing is necessary at this point, proceed immediately to the inactivation of endogenous peroxidases.)

### Inactivation of endogenous peroxidases

1. Incubate in H<sub>2</sub>O<sub>2</sub> (0,15% in methanol) for 30 min at RT

2. Alternative: incubate in 0.1 M HCl for 30-60 sec at RT, wash in 1 x PBS, incubate in H<sub>2</sub>O<sub>2</sub> (3% in MQ) for 10 min at RT
3. Wash filters well in excess MilliQ water
4. Wash 96% ethanol and let the filter air dry on Kimwipes/ Whatman paper\*.

### Hybridization in humidity chamber

Prepare a humidity chamber by inserting a tissue in a 50 ml tube and soak it with a formamide-water mix according to the formamide concentration of the hybridization buffer

1. Mix hybridization buffer with probe working solution [50 ng DNA  $\mu\text{l}^{-1}$ ] in a ratio 300:1;
2. Dip each filter completely into the hybridization solution and place filters face-up onto a parafilm covered glass slide; spread the rest of the solution evenly onto the filters; close tube firmly and keep the tube in a horizontal position (e.g. in an appropriate rack)
3. Incubate at 46 °C for 2-3 h (coastal water) or overnight for oligotrophic/open ocean water samples

### Washing

Transfer filter to the washing buffer by immersing the whole slide in the prewarmed washing buffer => filter will swim off

1. Wash filters in prewarmed washing buffer (10 min, 48 °C)
2. Transfer filters to 1 x PBS (do not let filter run dry!) and incubate for 15 min @ RT
3. To remove excess liquid, dab filter on blotting paper, but **do not let filter run dry**

### CARD

1. Prepare a fresh solution of H<sub>2</sub>O<sub>2</sub> (0,15% in PBS), keep it cool
2. Mix amplification buffer with H<sub>2</sub>O<sub>2</sub> solution in a ratio of 100:1 (as a guideline: the same volume as the hybridization mix is sufficient)
3. Add fluorescently labeled tyramide [1 mg dye ml<sup>-1</sup>] and mix well, keep in the dark

(The volume of labeled tyramide added strongly depends on the nature of the sample, start with 1:1000; if the signal is not sufficient: in-/decrease the ratio of added tyramide.)

4. Dip filter completely in the amplification mix, place filter sections face-up on a parafilm covered glass slide and put this e.g. into a petri dish; spread the rest of the amplification mix over the filters and incubate at 46 °C for up to 45 min. in the dark!
5. To remove excess liquid, dab filter on blotting paper and incubate in 1 x PBS for 5-10 min @ RT in the dark
6. Wash filters thoroughly in excess MQ and twice in 96% ethanol (~1 min), let completely air dry in the dark before counterstaining with DAPI
7. To keep the background fluorescence low, it is important to wash the filters in large volumes of water and ethanol

\* Filters may now be stored in the freezer without apparent loss in CARD-FISH signal intensity. RT: room temperature

### 3. CARD-FISH protocol for paraffin-embedded samples on slides (according to Blazejak et al., 2005, modified)

**please cite for this protocol:** Blazejak, A., C. Erseus, R. Amann, and N. Dubilier. 2005. *Coexistence of Bacterial Sulfide Oxidizers, Sulfate Reducers, and Spirochetes in a Gutless Worm (Oligochaeta) from the Peru Margin. Applied and Environmental Microbiology 71:1553-1561.*

**Attention: This protocol is for a hybridization temperature of 35°C (= add 20% more formamide in hybridisation buffer than for hybridization at 46°C!)**

#### Prehybridization treatments for paraffin-embedded samples

- Incubate slide as follows (in following order of steps:)
  1. RotiHistol® 100% 10 min RT
  2. RotiHistol® 100% 10 min RT
  3. RotiHistol® 100% 10 min RT
  4. Ethanol 95% 10 min RT
  5. Ethanol 80% 10 min RT
  6. Ethanol 70% 10 min RT
  7. HCl 0.2 M 12 min RT
  8. TrisHCl 20 mM 10 min RT
  9. Proteinase K 0.5 µg ml<sup>-1</sup> 5 min 37°C
  10. TrisHCl 20 mM 10 min RT
- Let air dry

(Incubations except for step 7, 9 and 10 might be shortened to 6 min.)

- Encircle each section with an immunostaining Pen (wax) to ensure that the solutions (hybridizations) do not run into each other.

#### Hybridization in humidity chamber

Prepare a humidity chamber by inserting a tissue to the bottom of a 50 ml tube and soak it with a formamide-water mix according to the formamide concentration of the hybridization buffer

1. Cover sections with hybridization buffer, insert slide into preheated humidity chamber, close tube firmly and keep the tube in a horizontal position (e.g. in an appropriate rack), incubate for 20 min at 35°C
2. remove buffer before the application of the new solution
3. Mix hybridization buffer with probe working solution [50 ng DNA µl<sup>-1</sup>] in a ratio 100:1 and apply onto sections
4. For each section 50 µl are sufficient
5. Incubate at 35°C for 3 h in horizontal position

## Washing

1. Remove hybridization buffer by dabbing the slide onto tissue; be careful to avoid mixing of different probes
2. (Quick) briefly dip the slide in first washing buffer
3. Wash the slide in second washing buffer for 15 min at 35 °C
4. Incubate slide in 1xPBS pH 8.0, shaking at RT for 15 min

Depending on background signal the incubation may last up to 1 h

## CARD

1. Prepare a new humidity chamber by inserting a tissue to the bottom of a 50 ml tube and soak with MQ
2. Prepare a fresh solution of H<sub>2</sub>O<sub>2</sub> (0,15% in PBS), keep it cool
3. Mix amplification buffer with H<sub>2</sub>O<sub>2</sub> solution in a ratio of 100:1 (as a guideline: the same volume as the hybridization mix is sufficient)
4. Add fluorescently labeled tyramide [1 mg dye ml<sup>-1</sup>] and mix well, keep in the dark

The volume of labeled tyramide added strongly depends on the nature of the sample, start with 1:1000; if the signal is not sufficient: in-/decrease the ratio of added tyramide.

5. Apply amplification mix onto sections and incubate at 37 °C for 20 min in the humidity chamber, keep dark!
6. To remove excess liquid, dab slide on blotting paper and incubate in 1 x PBS for 15 min at RT in the dark
7. Wash slide in MQ for 1 min at RT in the dark, let completely air dry before counterstaining with DAPI

#### 4. Preparation of buffers for CARD-FISH (for all three protocols)

##### Hybridization buffer (final volume 20 ml)

3.6 ml 5 M NaCl  
0.4 ml 1 M Tris HCl, pH 8.0  
20 µl SDS (20% w/v)  
x ml sterile dH<sub>2</sub>O (depending on probe, see Table A of standard FISH protocol)  
x ml formamide (depending on probe, see Table A standard FISH protocol)  
2.0 ml Blocking Reagent (10%, Roche, Basel; prepare according to manufacturer's instructions)  
2.0 g of dextran sulfate

Heat (40 to 60°C) and shake until the dextran sulphate has dissolved completely. Small portions of the buffer can then be stored at -20°C for several months.

##### Washing buffer (produce freshly when needed, final volume 50 ml)

0.5 ml 0.5 M EDTA, pH 8.0  
1.0 ml 1 M Tris HCl, pH 8.0  
x µl NaCl (depending on probe, see Table A standard FISH protocol)  
add dH<sub>2</sub>O to a final volume of 50 ml  
25 µl SDS (20% w/v)

The NaCl concentration in the washing buffer, as well as the formamide concentration of the hybridization buffer determines the stringency of the hybridization at the selected temperature.

##### CARD amplification buffer (final volume 40 ml)

2 ml of 20 × PBS, pH 7.6 (important for proper enzyme function)  
0.4 ml Blocking Reagent (10%, see above)  
16 ml 5 M NaCl  
add sterile dH<sub>2</sub>O to a final volume of 40 ml  
add 4 g of dextran sulfate

Heat (40 to 60°C) and shake until the dextran sulphate has dissolved completely. The amplification buffer can be stored in the refrigerator for several weeks.