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FISH Protocol: filter hybridization

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We use this protocol and it's working

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Abstract

FISH PROTOCOL FOR HYBRIDIZATION ON FILTERS
Filtration of water samples and paraformaldehyde fixation of microbial cells on polycarbonate-(PC)-filters.
For whole cell fixation, microbial cells should be concentrated from water samples by filtration on white polycarbonate-(PC)-filters using a vacuum filtration unit. After filtration of microbial cells on white PC-filters, all fixation and washing steps are performed in the vacuum filtration unit by successively applying and removing a vacuum.
In situ hybridization of PFA-fixed bacteria on a PC-filter is performed according to an established protocol (Glöckner *et al.* 1996, Pernthaler *et al.* 2001).

Before start

Reagents and equipment for filtration

- White polycarbonate (PC) filters (diameter 25 mm, pore size 0.2 µm; Millipore GTTP 02500, Eschborn, Germany)
- Cellulose-nitrate (CN) support filters (diameter 25 mm, pore size 0.45 µm, Sartorius)
- Vacuum filtration unit (Sartorius, Göttingen, Germany)
- Whatman 3M paper (Whatman Int. Ltd., Maidstone, UK)

Reagents for cell fixation

- Phosphate buffer (200 mM $Na_XH_XPO_4$)

200 mM NaH_2PO_4

200 mM Na_2HPO_4

adjust pH of solution 2 to 7.2-7.4 with solution 1

- 3x phosphate buffered saline (PBS) (390 mM NaCl, 15% (v/v) phosphate buffer, adjust pH to 7.2-7.4 with NaOH)
- 1x PBS
- 4% paraformaldehyde (PFA) solution.

For 50 ml PFA solution, heat 33 ml distilled water to 65°C; add 2 g PFA while stirring; add NaOH until the paraformaldehyde is dissolved; add 16.6 ml 3xPBS; let solution cool to room temperature; adjust pH to 7.2-7.4; filter the solution using syringes and 0.2 µm filters. Store the solution at -20°C. Caution: PFA is toxic. Wear gloves and a dust mask.

- 96% (v/v) ethanol
- Ice

Reagents and equipment for hybridization and epifluorescence microscopy

- 50 ml screw-cap plastic tubes
- 50%, 80%, and 96% (v/v) ethanol
- Double distilled water (ddH_2O)
- 5 M NaCl
- Tris buffer (1 M Tris/HCl, pH 8.0)
- Formamide (toxic, wear gloves and work in a fume hood especially when handling warm hybridisation buffer that contains formamide). Note: Purchase the highest quality of formamide (molecular biology grade). Lower grade formamide can be contaminated with cations that reduce hybridisation stringency.
- 10% (w/v) sodium dodecyl sulphate (SDS)
- 5 M ethylenediaminetetraacetate (EDTA), pH 8.0
- Fluorescently labelled rRNA-targeted oligonucleotide probes (probe concentration should be 30 ng/µl of probes labelled with Cy3 and Cy5 and 50 ng/µl of probes labelled with FLUOS). Probe solutions must be stored in the dark at -20°C. Unlabelled competitor oligonucleotides (same concentration as corresponding probe)
- Ice-cold ddH_2O
- Antifadent (e.g., Citifluor AF1, Citifluor Ltd., London, UK)
- Hand-warm 0.5-1% (w/v) agarose
- DNA stains: SYBR Green I (FMC Bioproducts, Rockland, USA)

1.5 µM 4',6-diamidino-2-phenylindole (DAPI) solution

- Standard microscopic slides and cover slips
- Hybridisation buffer : 180 µl 5 M NaCl

20 µl Tris buffer
formamide and ddH_2O (both to be varied depending on stringency)
1 µl 10% SDS

- Washing buffer: 1 ml Tris buffer

5 M NaCl
0.5 M EDTA
top up with ddH_2O to 50 ml

1 Sterilize filtration unit and other equipment.

2 Moisten CN support filters and PC filters (shiny side of the PC-filter should face upwards) in sterile water.

3 Place moistened CN support filters and PC filters (shiny side up) in the filtration unit.

4 Check planarity of filtration unit and filters with a water-level to allow for an even distribution of planktonic cells on the filter surface.

5 Filter water sample by applying a gentle vacuum (approx. 150 mbar). 100 ml of water is sufficient for cell numbers of around 10^5 ml^{-1}.

100 mL water sample

Command

150 mbar

VACUUM FILTRATION

6 Remove vacuum and overlay filter with 4 ml of 3% PFA fixative (mix 3 ml of 4% PFA solution with 1 ml 1x PBS) for 20 min.

4 mL 3% PFA fixative

00:20:00 fixation

7 Remove fixative by applying a vacuum.

8 Wash cells with 3 ml 1x PBS.

3 mL 1xPBS

9 Wash cells with sterile water.

10 Repeat washing with 1x PBS and water two times.
3 mL 1xPBS

11 Remove filter from filtration unit with sterile tweezers and place it on a Whatman paper for drying (shiny side up) in a dust-free environment (e.g. petri dish).

12 Label shiny side of the filter with a pencil and store at -20°C until hybridization. Filters can be stored frozen for several months without apparent loss of hybridization signal.

Negative control: Repeat procedure with sterile water.

Fluorescence in situ hybridization

13 Prepare 2 ml of hybridization buffer.
2 mL hybridization buffer

14 Add 3 µl of probe working solution to 27 µl of hybridization buffer; keep probe solutions dark and on ice.
3 µL probe working solution
27 µL hybridization buffer

15 Place filter on a microscopic slide (shiny side up) and cut it with a sterile scalpel in four sections.

16 Label filter sections with a pencil.

17 Place filter section(s) on a microscopic slide (shiny side up); several filter sections can be placed on one slide and hybridized simultaneously with the same probe.

18 For the preparation of an equilibrated chamber, place a piece of tissue into a 50 ml tube and moisten it with the remaining hybridization buffer.

19 Drop 30 ml hybridization solution on a filter section and place the slide horizontally into the 50 ml tube and close the lid tightly.
30 mL hybridization solution

20 Incubate at 46°C for at least 90 min in a hybridization oven.
46 °C hybridization oven
01:30:00 hybridization

21 Prepare 50 ml of washing buffer in a 50 ml plastic tube and pre-heat at 48°C.
50 mL washing buffer
48 °C pre-heating

22 Quickly transfer filter sections into warm washing buffer and incubate for 15 min at 48°C in the water bath.
48 °C water bath
00:15:00 incubation

23 Pick filter sections with sterile tweezers and rinse in sterile double-distilled water for several seconds.

24 Dry filter sections on Whatman paper.

25 For counterstaining with a nucleic acid dye, place filter sections on a slide, cover with 80 µl of SYBR Green I (Schmid *et al.* 2000) or DAPI solution (Hicks *et al.* 1992), and incubate for 10 minutes on ice in the dark. Wash filter sections for several seconds in cold 50% ethanol, rinse in double-distilled water, and dry on Whatman paper.
80 µL SYBR Green I
0 °C on ice
00:10:00 incubation in the dark

26 For microscopy, place dry filter section on a glass slide and apply one to two drops of antifadent on the filter.

27 Put a microscope cover slip on top and wait until the antifadent has spread over the filter.
NOTE: too much antifadent can blur the microscope image, avoid bubbles.

28 Observe the sample under an epifluorescence microscope or confocal laser scanning microscope equipped with suitable filters or lasers, respectively.

29 Slides embedded in antifadent can be stored at 4°C (do not freeze) for several days before the probe-conferred fluorescence begins to decline. Alternatively, the antifadent can be removed with water and the dried slides can be stored at -20°C for longer periods.

NOTE: Probe-conferred fluorescence fades much more rapidly than DAPI fluorescence.

30 **Microscopy and manual quantification of absolute and relative cell numbers.** Absolute total cell numbers are determined by manual counting of DAPI-stained cells in at least 50 randomly

chosen microscopic sections. For analyses of relative cell numbers of individual bacterial populations, DAPI-staining is combined with FISH. For each hybridization experiment, DAPI- and probe-stained cells in 20 randomly chosen microscopic sections are counted at a magnification of x400 respectively x1000. (NOTE for weak fluorescence signals: To avoid fluorescence bleaching of probe-labeled cells during DAPI examination, each microscopic field should be first viewed with the FLUOS filter, thereafter with the Cy3 filter before finally switching to the DAPI filter). At least 500 DAPI-stained cells should be counted to obtain a counting error < ±5%. All oligonucleotide probe counts are corrected by subtracting the counts obtained with the negative control probe NON338. The ratio of the number of those cells labeled by the rRNA-targeted oligonucleotide probe to the total number of bacterial cells stained by DAPI is calculated for each microscopic section.