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## PhageFISH detailed protocol

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**Protocol status:** Working

**We use this protocol and it's working**

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## Abstract

This protocol details about PhageFISH protocol.

## Attachments



**627-1301.docx**

32KB

## Guidelines

### Controls to consider:

- Faecal sample with no target for the phage probe

### Timeframe:

Day 1	3h20m
Prepare samples	<b>30 minutes*</b>
Fix samples	<b>1 hour + 10 min</b> (1h incubation)
<i>Prepare <u>permeabilisation buffer and HCl</u></i>	<b>10-15 min</b>
<i>Prepare ice for <u>permeabilisation</u></i>	<b>5 min</b>
Wash	<b>5 min</b> ❄️
Permeabilise cells	<b>1 hour + 10 min</b> (1h incubation)
Wash	<b>10 min</b>
Inactivate peroxidases	<b>15 min</b> (10 min incubation)
Wash	<b>5 min</b> ❄️
<b>Day 2</b>	<b>6h5m</b>
Prepare probes	<b>15 min</b>
Hybridisation of cyanine-labelled probes	<b>3 hours + 10 min</b> (3h incubation)
Wash	<b>20 min</b> (15 min incubation) ❄️
Pre-hybridisation of DIG-labelled probes	<b>1 hour + 15 min</b> (1h incubation)
<i>Prepare probes</i>	<b>20 min</b>
Hybridisation of DIG-labelled probes	<b>1 hour + overnight</b> (1h incubation) 🌙
<b>Day 3</b>	<b>5h35m</b>
Wash	<b>2 hours + 15 min</b> (30min + 1.5h incubation)
<i>Prepare antibody washing and CARD buffers</i>	<b>30 min</b>
Antibody binding	<b>2 hours + 15 min</b> (30min + 1.5h incubation)
Wash	<b>35 min</b>
CARD amplification	<b>1 hour</b> (45 min incubation)
Wash	<b>30 min</b> ❄️
<b>Day 4</b>	<b>1-6h</b>
Staining and sealing slides	<b>1 hour*</b> ❄️
Microscopy	<b>1-5 hours*</b>
<b>Total:</b>	<b>17 hours (not incl. microscopy)</b> (approx. 12 hours incubation time) <b>3-5 days</b>




\*depending on number of samples

❄️ Freezing and stopping possible after step

🌙 Overnight incubation after step

## Materials

### Necessary materials:

- Poly-L-lysine coated glass slides with writing area
- Pencil for writing (DO NOT use sharpie)
- Pipette tip lids for holding glass slides (one will fit four slides, collect one lid for each condition tested)
- Humidity chambers (one for each formamide concentration used simultaneously). Anaerobic growth chambers work well.
- Aluminium foil (to protect samples from light)
- Ice
- Fume hood
- Incubator set to  46 °C
- Incubator (or oven) set to  85 °C
- Water bath set to  48 °C
- Optimised and diluted Cy-labelled probes (see *Optimisation of formamide concentration*)
- Diluted phage probes (see *Buffers and Reagents*)
- All buffers (see *Buffers and Reagents*)
- Faecal samples of interest

### Note


- If possible, samples should be submerged in plenty of buffer. Four slides can be submerged in 30-50ml in a pipette tip lid. For washing, very light agitation could be used (e.g. the shaking incubator set to 25rpm).
- For valuable solutions (like probe-solutions), only cover the sample area and handle with care. Use 500µl-1ml to cover sample area.
- All incubations are at room temperature unless specified.
- DO NOT allow samples to dry unless specified.
- When working with paraformaldehyde and formamide always work in the hood.
- After using humidity chambers, allow fumes to evaporate in fume hood overnight.


## Before start

Prepare buffers (see **Preparation of Buffers for PhageFISH protocol**).



## Fix faecal samples to glass slides

1 Mix a loopful faecal sample with  10-20  $\mu\text{L}$  PBS (1X) and vortex thoroughly. 



2 Allow suspension to settle for  00:05:00 to avoid large debris. 

3 Take  10  $\mu\text{L}$  of the supernatant and place on coated glass slide.

4 Smear the droplet thinly over the slide using a cover slip.


### Note



Avoid smearing all the way to the edges.

5 Allow the sample to dry – this should not take more than  00:10:00 . 


### Note

If not dry after 10 minutes, aspirate off excess liquid.

6 Work in fume hood. Overlay the slides with 1% paraformaldehyde (PFA). Ensure the whole sample area is covered (approx.  1 mL ).


7 Incubate for  01:00:00 at  Room temperature in the fume hood. 

### Note

This incubation should NOT exceed  01:00:00 ! 

8 Aspirate off excess PFA.




9 Wash in PBS for  00:01:00 .

1m

#### Note

If a lot of PFA remains on the sample, rinse twice in PBS.

**FREEZING POINT** – if necessary, samples can be rinsed in sterile water and 96% ethanol and air dried before freezing in closed box covered with aluminium foil at  -20 °C .





## Permeabilise cells

10 Add lysozyme to permeabilisation buffer.



11 Overlay samples with permeabilisation buffer.

12 Incubate  On ice for  01:00:00 .

1h



13 Discard permeabilisation buffer.

14 Wash samples in PBS for  00:05:00 .

5m



15 Wash samples in sterile water for  00:01:00 .

1m



## Inactivate peroxidases

16 Incubate samples in  0.01 Molarity (M) HCl for  00:10:00 .

10m



17 Wash samples in PBS for  00:05:00 .


5m



18 Wash samples in sterile water for  00:01:00 .

1m




19 Wash samples in 96% ethanol for  00:01:00 .

1m




20 Allow slides to dry on blotting paper or filter paper.

Note

**FREEZING POINT** – if necessary, samples can be frozen after drying. Store in closed container covered with aluminium foil at  -20 °C .

### Cy-labelled probe hybridisation (16S rRNA probes)


21 Work in fume hood. Place a paper towel in the bottom of the hybridisation chamber and soak in formamide/milliQ solution corresponding to the hybridisation buffer concentration.



22 Overlay samples with hybridisation buffer-probe mix at  0.5 ng/μl of each probe and close humidity chamber.

23 Incubate at  46 °C for  03:00:00 .

3h



24 Prepare the washing buffer – heat to  48 °C .

25 Work in fume hood. Overlay the samples with washing buffer and incubate for  00:15:00 at  48 °C (in humidity chamber to avoid formamide fumes).

15m



26 Wash samples in sterile water.



27 Allow samples to dry.

Note

**FREEZING POINT** – if necessary, samples can be frozen after drying. Store in closed container covered with aluminium foil at  $-20\text{ }^{\circ}\text{C}$ .

## Phage probe hybridisation

28 Work in fume hood. Place a paper towel in the bottom of the hybridisation chamber and soak in formamide/milliQ solution corresponding to the hybridisation buffer concentration.

29 Overlay samples with hybridisation buffer (no probes!) and close humidity chamber (  $500\text{ }\mu\text{L}$  per slide).

30 Incubate for  $01:00:00$  at  $46\text{ }^{\circ}\text{C}$ .

1h



31 Cover the samples with hybridisation buffer-probe mix at  $10\text{ }\mu\text{g}/\mu\text{l}$  of each probe ( $500\mu\text{l}$  per slide).

32 Place the dish back in the humidity chamber and incubate for  $01:00:00$  at  $85\text{ }^{\circ}\text{C}$ .

1h



33 Immediately place the humidity chamber at hybridisation temperature **Overnight**.

1h



34 Wash slides.



34.1 Wash slides in gene washing buffer I for  $00:01:00$ . (1/3)




1m

34.2 Wash slides in gene washing buffer I for  $00:01:00$ . (2/3)

1m



- 34.3 Wash slides in gene washing buffer I for 00:01:00 . (3/3) 1m
- 34.4 Wash slides in gene washing buffer I for 00:30:00 at 42 °C . 30m
- 35 Wash slides.
- 35.1 Wash slides in gene washing buffer II for 00:01:00 . (1/3) 1m
- 35.2 Wash slides in gene washing buffer II for 00:01:00 . (2/3) 1m
- 35.3 Wash slides in gene washing buffer II for 00:01:00 . (3/3) 1m
- 35.4 Wash slides in gene washing buffer II for 01:30:00 at 42 °C . 1h 30m
- 36 Wash slides in PBS for 00:01:00 . 1m
- 
- Antibody binding**
- 37 Cover slides with antibody-blocking solution. Incubate for 00:30:00 . 30m
- 
- 38 Discard antibody-blocking solution and cover with antibody binding solution. Incubate for 01:30:00 . 1h 30m
- 
- 39 Wash slides.
- 39.1 Wash slides in antibody washing solution for 00:01:00 . 1m

- 39.2 Wash slides in antibody washing solution for  00:10:00 . (1/3) 10m
- 39.3 Wash slides in antibody washing solution for  00:10:00 . (2/3) 10m
- 39.4 Wash slides in antibody washing solution for  00:10:00 . (3/3) 10m







## CARD amplification

- 40 Mix  1 mL amplification buffer with  10  $\mu$ L H<sub>2</sub>O<sub>2</sub> and  2  $\mu$ L Alexa tyramides (488). Vortex to mix. 
- 41 Cover slides with CARD buffer-tyramide mix (approx.  500  $\mu$ L per slide). Incubate at  37 °C for  00:45:00 . 45m 
- 42 Wash slides. 
- 42.1 Wash slides in PBS for  00:01:00 . 1m
- 42.2 Wash slides in PBS for  00:05:00 . 5m
- 42.3 Wash slides in PBS for  00:10:00 at  46 °C . 10m
- 42.4 Wash slides in PBS for  00:10:00 at  46 °C . 10m
- 43 Wash slides in sterile water for  00:01:00 . 1m 
- 44 Wash slides in 96% ethanol for  00:01:00 . 1m

Note

**FREEZING POINT**

## Staining and embedding

- 45 Mix  1 mL SlowFade Gold antifade reagent with 1 5m/ml DAPI (final concentration  5 µg/mL ), can be stored at  Room temperature ). 
- 46 Place  10 µL solution in small droplets on the slides.
- 47 Place coverslip and press down gently to remove air pockets without disturbing the sample area.
- 48 Seal edges with clear nail polish.
- 49 Samples can now be stored at  -20 °C in covered container indefinitely.