Feb 01, 2023

PhageFISH detailed protocol

PLOS One

Peer-reviewed method

In 1 collection

DOI
dx.doi.org/10.17504/protocols.io.4r3l273wqg1y/v1

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DOI: dx.doi.org/10.17504/protocols.io.4r3l273wqg1y/v1

Protocol Citation: Line Jensen Ostenfeld, Saria Otani 2023. PhageFISH detailed protocol. **protocols.io https://dx.doi.org/10.17504/protocols.io.4r3l273wqg1y/v1**

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

Created: January 27, 2023

Last Modified: February 01, 2023

Protocol Integer ID: 75967

Keywords: Staining and embedding, CARD amplification, Phage probe hybridisation, Antibody binding

Funders Acknowledgement:
NovoNordisk
Grant ID: NNF16OC0021856

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	**30 minutes***
Fix samples	**1 hour + 10 min** (1h incubation)
Prepare permeabilisation buffer and HCl	*10-15 min*
Prepare ice for permeabilisation	*5 min*
Wash	**5 min** ❄
Permeabilise cells	**1 hour + 10 min** (1h incubation)
Wash	**10 min**
Inactivate peroxidases	**15 min** (10 min incubation)
Wash	**5 min** ❄

Day 2	**6h5m**
Prepare probes	**15 min**
Hybridisation of cyanine-labelled probes	**3 hours + 10 min** (3h incubation)
Wash	**20 min** (15 min incubation) ❄
Pre-hybridisation of DIG-labelled probes	**1 hour + 15 min** (1h incubation)
Prepare probes	*20 min*
Hybridisation of DIG-labelled probes	**1 hour + overnight** (1h incubation) ☽

Day 3	**5h35m**
Wash	**2 hours + 15 min** (30min + 1.5h incubation)
Prepare antibody washing and CARD buffers	*30 min*
Antibody binding	**2 hours + 15 min** (30min + 1.5h incubation)
Wash	**35 min**
CARD amplification	**1 hour** (45 min incubation)
Wash	**30 min** ❄

Day 4	**1-6h**
Staining and sealing slides	**1 hour*** ❄
Microscopy	**1-5 hours***
Total:	**17 hours (not incl. microscopy)** (approx. 12 hours incubation time) **3-5 days**

***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 µL PBS (1X) and vortex thoroughly.

2 Allow suspension to settle for 00:05:00 to avoid large debris. 5m

3 Take 10 µL 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 . 10m

> **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. 1h

> **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 [M] 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 °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 µL per slide).

30 Incubate for 01:00:00 at 46 °C. 1h

31 Cover the samples with hybridisation buffer-probe mix at 10 pg/µl of each probe (500µl per slide).

32 Place the dish back in the humidity chamber and incubate for 01:00:00 at 85 °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 µL H_2O_2 and 2 µL Alexa tyramides (488). Vortex to mix.

41 Cover slides with CARD buffer-tyramide mix (approx. 500 µL 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.