**Permeabilisation buffer:**

50 ml:

* PBS [pH 7.5] (10 x) 5 ml
* Tris-HCl [pH 8.0] (1 M) 5 ml
* EDTA (0.5) 5 ml
* Water 35 ml
* Lysozyme

Permeabilisation mix: Mix PBS, Tris-HCl, EDTA, and water.

High conc. lysozyme buffer: Dissolve lysozyme in appropriate buffer volume (50 mg lysozyme to 10 ml buffer). It may be necessary to heat the solution to 37°C.

Dilute lysozyme buffer into large buffer volume by adding 1 part lysozyme buffer to 9 parts permeabilisation mix.

Final concentration:

1 x PBS

0.1 M Tris-HCl

0.05 M EDTA

0.5 mg/ml Lysozyme

Permeabilisation buffer should not be stored.

Prepare in aliquots of 1 ml.

**rRNA hybridisation buffer**

40 ml:

* Dextran sulphate 4 g
* NaCl (5 M) 7.2 ml
* Tris-HCl [pH 8.0] (1 M) 0.8 ml
* Water 4 ml
* Nucleic acid blocking reagent (10%) 4 ml
* Sheared salmon sperm (10 mg/ml) 1 ml
* Yeast RNA (10 mg/ml) 1 ml
* Formamide (100%) 17.5 ml
* SDS (20%) 40 µl

Mix dextran sulphate, NaCl, Tris-HCl, and water in a falcon tube and vortex or shake thoroughly to disperse dextran sulphate. Heat solution in waterbath at 37°C-48°C and vortex until dextran sulphate is completely dissolved.

Cool the solution down to room temperature.

Add nucleic acid blocking agent, sheared salmon sperm, yeast RNA, formamide, and SDS. Adjust volume with water to reach 40 ml if necessary.

Vortex to mix.

Spin down solution briefly and filter through 0.22µm syringe filter.

Final concentration:

10% dextran sulphate

0.9 M NaCl

20 mM Tris-HCl

1% nucleic acid blocking reagent

0.25 mg/ml sheared salmon sperm

0.25 mg/ml yeast RNA

35% formamide

0.02% SDS

Store in aliquots at -20°C. Reheat to 37°C before use to redissolve precipitate.

Prepare several in aliquots of 900µ

**rRNA hybridisation wash buffer:**

50 ml:

* \*NaCl (5 M) 700 µl
* \*EDTA [pH 8.0] (0.5 M) 500 µl
* Tris-HCl (1 M) 1 ml
* Water up to 50 ml
* SDS (20%) 25 µl

Mix \*NaCl, \*EDTA, and Tris-HCl in 50 ml falcon tube.

Add water up the 50 ml mark.

Add SDS.

Final concentrations:

70 mM NaCl

5 mM EDTA

20 mM Tris-HCl

0.01% SDS

\* NOTE: Na+ concentrations depend on the amount of formamide used in the hybridisation buffer. The formamide concentration is calculated based on probe properties to achieve a hybridisation temperature of 42°C-50°C

The formamide (FA) concentrations and the corresponding Na+ ions concentrations when washing at 48°C are as follows:

0% FA — 900 mM Na+

5%FA— 636 mM Na+

10% FA — 450 mM Na+

15% FA — 318 mM Na+

20% FA— 225 mM Na+

25% FA— 159 mM Na+

30% FA — 112 mM Na+

35% FA — 80 mM Na+

40% FA — 56 mM Na+

45% FA—40 mM Na+

50% FA— 28 mM Na+

55% FA — 20 mM Na+

60% FA — 14 mM Na+

Prepare in aliquots of 50 – prepare at least two aliquots per cycle

**rRNA CARD buffer**

40 ml:

* Dextran sulphate 4 g
* PBS [pH 7.4] (10 x) 4 ml
* NaCl (5 M) 16 ml
* Water up to 40 ml
* Nucleic acid blocking reagent (10%) 400 µl

Mix dextran sulphate, PBS, and NaCl. Add water up to 40 ml. vortex thoroughly to disperse dextran sulphate. Heat solution in waterbath at 37°C-48°C and vortex until dextran sulphate is completely dissolved.

Allow solution to cool down to room temperature and add nucleic acid blocking reagent.

Vortex to mix.

Spin down briefly.

Filter through 0.22 µm syringe filter.

Final concentration:

1 x PBS

10% dextran sulphate

0.1% nucleic acid blocking reagent

2 M NaCl

Store in aliquots at 4°C. Reheat to 37°C before use to redissolve precipitate.

Prepare in aliquot of 3 ml

**Gene hybridisation buffer**

40 ml:

* Dextran sulphate 4 g
* SSC (20 x) 10 ml
* EDTA [pH 8.0] (0.5 M) 1.6 ml
* Water 4.4 ml
* Nucleic acid blocking reagent (10%) 4 ml
* Sheared salmon sperm (10 mg/ml) 1 ml
* Yeast RNA (10 mg/ml) 1 ml
* Formamide (100%) 14 ml
* SDS (20%) 200 µl

Mix dextran sulphate, SSC, EDTA, and water in a falcon tube and vortex or shake thoroughly to disperse dextran sulphate. Heat solution in waterbath at 37°C-48°C and vortex until dextran sulphate is completely dissolved.

Cool the solution down to room temperature.

Add nucleic acid blocking agent, sheared salmon sperm, yeast RNA, formamide, and SDS.

Vortex to mix.

Spin down solution briefly and filter through 0.22µm syringe filter.

Final concentration:

35% formamide

5 x SSC

10% dextran sulphate

0.1% SDS

20 mM EDTA

1% nucleic acid blocking reagent

0.25 mg/ml sheared salmon sperm

0.25 mg/ml yeast RNA

Store in aliquots at -20°C. Reheat to 42°C before use to redissolve precipitate.

**Gene hybridisation wash buffer I**

50 ml:

* SSC (20 x) 5 ml
* SDS 250 µl
* Water up to 50 ml

Mix SSC and water in a 50 ml falcon tube.

Add SDS.

Vortex to mix.

Final concentration:

2 x SSC

0.1% SDS

Store for 1-2 days at 42°C

**Gene hybridisation wash buffer II**

50 ml:

* SSC (20 x) 250 µl
* SDS 250 µl
* Water up to 50 ml

Mix SSC and water in a 50 ml falcon tube.

Add SDS.

Vortex to mix.

Final concentration:

0.1 x SSC

0.1% SDS

Store for 1-2 days at 42°C

**Gene CARD amplification buffer**

40 ml:

* Dextran sulphate 8 g
* PBS [pH 7.4] (10 x) 4 ml
* NaCl (5 M) 16 ml
* Water 15.6 ml
* Nucleic acid blocking reagent (10%) 400 µl

Mix dextran sulphate, PBS, NaCl, and water.

Vortex or shake thoroughly to disperse dextran sulphate. Heat solution in waterbath at 37°C-48°C and vortex until dextran sulphate is completely dissolved.

Allow solution to cool down to room temperature and add nucleic acid blocking reagent.

Vortex to mix.

Spin down briefly.

Filter through 0.22 µm syringe filter.

Final concentrations:

1 x PBS

20% dextran sulphate

0.1% blocking reagent

2 M NaCl

Store in aliquots at 4°C. Reheat to 37°C before use to redissolve precipitate.

PhageFISH for DIG-labelled bacterial probes

1. Fix liquid samples to glass slides
	1. Place liquid sample in a 30-50µl droplet on poly-L-lysine coated slide
	2. Dry in warm incubator for approx. 30 minutes or until the droplet has dried out
	3. OPTIONAL: if sample is very dilute add several droplets and repeat drying procedure
	4. Add 1% paraformaldehyde to cover the sample area
	5. Incubate at room temperature for 1 hour
	6. Aspirate the paraformaldehyde off
	7. Rinse samples in PBS for 1 minute
2. Fix faecal samples to glass slides
	1. Mix a small faecal sample with 10-20µl PBS (1X) and vortex thoroughly
	2. Allow suspension to settle for 5 minutes
	3. Take 10µl of the supernatant and place on coated glass slide
	4. Smear the droplet over the slide using a cover slip
	5. Allow the sample to dry – this should not take more than 10 minutes
	6. Overlay the slides with 1% paraformaldehyde. Ensure the whole sample area is covered (approx. 1ml)
	7. Incubate for 1 hour at room temperature or overnight at +4°C
	8. Aspirate off excess paraformaldehyde
	9. Wash in PBS for 1 minute

FREEZING POINT

1. Permeabilise cells
	1. Add lysozyme to permeabilisation buffer
	2. Overlay samples with permeabilisation buffer
	3. Incubate on ice for 1h
	4. Wash samples in PBS for 5 minutes
	5. Wash samples in sterile water for 1 minute
2. Inactivate peroxidases
	1. Incubate samples in 0.01M HCl for 10 minutes
	2. Wash samples in PBS for 5 minutes
	3. Wash samples in sterile water for 1 minute
	4. Wash samples in 96% ethanol for 1 minute
	5. Allow slides to dry on blotting paper or filter paper
3. rRNA hybridisation of DIG-labelled probes
	1. Place filters in a petri dish and spot up to 100µl hybridisation buffer to cover the filters
	2. Transfer to a humidity chamber with hybridisation buffer soaked paper towels
	3. Incubate for 1 hour at hybridisation temperature\_\_\_\_\_\_.
	4. Mix 1ml gene hybridisation buffer with 1µl of each probe. Vortex to mix
	5. Place one droplet of 30-100µl probe mix on a petri dish for each filter
	6. Place the filters face down in the probe mix droplets
	7. Place the dish back in the humidity chamber and incubate for 1 hour at 85°C
	8. Immediately place the humidity chamber at hybridisation temperature overnight
	9. Wash filters in gene washing buffer I for 3x1 minute and then 30 minutes at 42°C
	10. Wash filters in gene washing buffer II for 3x1 minute and then 1.5 hours at 42°C
	11. Wash filters in PBS for 1 minute
4. Antibody binding
	1. Place filters in a petri dish and add antibody blocking solution to cover the filters. Incubate for 30 minutes
	2. Move filters to antibody binding solution and incubate for 1.5 hours
	3. Wash filters in antibody washing solution for 1 minute and then 3X10 minutes
5. CARD amplification
	1. Mix 1ml amplification buffer with 10µl H2O2 and 2µl Alexa tyramides (488). Vortex to mix.
	2. Place filters in a petri dish and cover with probe mix by spotting droplets of 30-100µl
	3. Wash filters in PBS for 1 minute, then 5 minutes and 2x10 minutes at 46°C
	4. Wash filters in sterile water for 1 minute
	5. Wash filters in 96% ethanol for 1 minute
6. Remove RNases
	1. Add 10.8ml sterile water, 1.2ml Tris-HCl (1M, pH 8), 15µl RNase I, and 30µl RNase A to a 15ml falcon tube
	2. Place filters in the RNase solution and incubate for 1 hour at 37°C
	3. Wash filters in PBS for 5 minutes
	4. Repeat wash
	5. Wash filters in sterile water for 1 minute
7. Gene hybridisation
	1. Cover samples with hybridisation buffer
	2. Transfer to a humidity chamber with formamide soaked paper towels at the corresponding concentration
	3. Incubate for 1 hour at hybridisation temperature (approx. 46°C)
	4. Mix 1ml gene hybridisation buffer with 1µl of each probe. Vortex to mix
	5. Cover the samples with the hybridisation buffer-probe mix
	6. Place the dish back in the humidity chamber and incubate for 1 hour at 85°C
	7. Immediately place the humidity chamber at hybridisation temperature overnight

OVERNIGHT

* 1. Wash filters in gene washing buffer I for 3x1 minute and then 30 minutes at 42°C
	2. Wash filters in gene washing buffer II for 3x1 minute and then 1.5 hours at 42°C
	3. Wash filters in PBS for 1 minute
1. Antibody binding
	1. Place filters in a petri dish and add antibody-blocking solution to cover the filters. Incubate for 30 minutes
	2. Move filters to antibody binding solution and incubate for 1.5 hours
	3. Wash filters in antibody washing solution for 1 minute and then 3X10 minutes
2. CARD amplification
	1. Mix 1ml amplification buffer with 10µl H2O2 and 2µl Alexa tyramides (594). Vortex to mix.
	2. Place filters in a petri dish and cover with probe mix by spotting droplets of 30-100µl

Incubate at 37°C for 45 minutes

* 1. Wash filters in PBS for 1 minute, then 5 minutes and 2x10 minutes at 46°C
	2. Wash filters in sterile water for 1 minute
	3. Wash filters in 96% ethanol for 1 minute

OPTIONAL FREEZING POINT

1. Staining
	1. Mix 1ml SlowFade Gold with 1µl 5mg/ml DAPI dye.
	2. Apply 5-10µl mix in droplets to each slide.
	3. Apply coverglass and carefully press down to seal sample with minimal air bubbles.
	4. Seal with clear nail polish on all edges of the sample.
	5. Allow to cure completely.
	6. Store at -20°C.

PhageFISH detailed protocol

**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

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.

Controls to consider:

* Faecal sample with no target for the phage probe

Before starting:

Prepare buffers (see *Buffers and Reagents*)

**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

**Protocol:**

1. Fix faecal samples to glass slides
	1. Mix a loopful faecal sample with 10-20µl PBS (1X) and vortex thoroughly
	2. Allow suspension to settle for 5 minutes to avoid large debris
	3. Take 10µl of the supernatant and place on coated glass slide
	4. Smear the droplet thinly over the slide using a cover slip (avoid smearing all the way to the edges)
	5. Allow the sample to dry – this should not take more than 10 minutes (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. 1ml).
	7. Incubate for 1 hour at room temperature in the fume hood. This incubation should NOT exceed 1 hour!
	8. Aspirate off excess PFA
	9. Wash in PBS for 1 minute (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.

1. Permeabilise cells
	1. Add lysozyme to permeabilisation buffer
	2. Overlay samples with permeabilisation buffer
	3. Incubate on ice for 1h
	4. Discard permeabilisation buffer
	5. Wash samples in PBS for 5 minutes
	6. Wash samples in sterile water for 1 minute
2. Inactivate peroxidases
	1. Incubate samples in 0.01M HCl for 10 minutes
	2. Wash samples in PBS for 5 minutes
	3. Wash samples in sterile water for 1 minute
	4. Wash samples in 96% ethanol for 1 minute
	5. Allow slides to dry on blotting paper or filter paper

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

1. Cy-labelled probe hybridisation (16S rRNA probes)
	1. 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.
	2. Overlay samples with hybridisation buffer-probe mix at 0.5ng/µl of each probe and close humidity chamber.
	3. Incubate at 46°C for 3 hours
	4. Prepare the washing buffer – heat to 48°C
	5. Work in fume hood. Overlay the samples with washing buffer and incubate for 15 minutes at 48°C (in humidity chamber to avoid formamide fumes)
	6. Wash samples in sterile water
	7. Allow samples to dry

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

1. Phage probe hybridisation
	1. 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.
	2. Overlay samples with hybridisation buffer (no probes!) and close humidity chamber (500µl per slide).
	3. Incubate for 1 hour at 46°C
	4. Cover the samples with hybridisation buffer-probe mix at 10pg/µl of each probe (500µl per slide).
	5. Place the dish back in the humidity chamber and incubate for 1 hour at 85°C
	6. Immediately place the humidity chamber at hybridisation temperature **overnight**
	7. Wash slides in gene washing buffer I for 3x1 minute and then 30 minutes at 42°C
	8. Wash slides in gene washing buffer II for 3x1 minute and then 1.5 hours at 42°C
	9. Wash slides in PBS for 1 minute
2. Antibody binding
	1. Cover slides with antibody-blocking solution. Incubate for 30 minutes
	2. Discard antibody-blocking solution and cover with antibody binding solution. Incubate for 1.5 hours
	3. Wash slides in antibody washing solution for 1 minute and then 3X10 minutes
3. CARD amplification
	1. Mix 1ml amplification buffer with 10µl H2O2 and 2µl Alexa tyramides (488). Vortex to mix.
	2. Cover slides with CARD buffer-tyramide mix (approx. 500µl per slide). Incubate at 37°C for 45 minutes
	3. Wash slides in PBS for 1 minute, then 5 minutes and 2x10 minutes at 46°C
	4. Wash slides in sterile water for 1 minute
	5. Wash slides in 96% ethanol for 1 minute

FREEZING POINT

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