PhageFISH PROTOCOL FOR (I) COMBINED PHAGE GENE DETECTION WITH rRNA DETECTION FOR THE IDENTIFICATION OF HOST CELLS, AND (II) DETECTION OF FREE PHAGE PARTICLES

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BEFORE STARTING THE phageFISH EXPERIMENT: PROBE DESIGN, PROBE SYNTHESIS AND HYBRIDIZATION STRINGENCY

To target the phage gene of interest, prepare 4 to 6 dsDNA polynucleotide probes (300 bp each). Use NonPoly350Probe (Moraru *et al.*, 2010) as gene negative control. Probes (with Dig) are synthesized by incorporating Dig-dUTP into dsDNA via PCR (70 µM Dig-dUTP), using the PCR Dig Probe Synthesis Kit (Roche, cat. no. 11636090910) according to the manufacturer's instructions. Targets (i.e. PCR-amplified 300 bp target regions of the phage gene without Dig) are synthesized similarly as the probes, but without Dig-UTP in the PCR mix. The resulting PCR products are column-purified using the Gene Clean Turbo kit (Q-Biogene, cat. no. 1102-600), eluted in 1x TE, and checked electrophoretically in 2% agarose gels, for size and Dig incorporation. Run the gels at 100 V for 30 - 45 min at 500 mA using a 1-kb size ladder. The Dig-labeled probes are at a higher molecular weight and therefore run at a higher position in the gel than the respective targets, indicating Dig incorporation. The concentration of PCR products (probes and targets) are best determined using a Quant-iT^{TM}Picogreen assay (Invitrogen, cat. no. P7589) or spectrophotometrically using a NanoDrop instrument. The probes were stored at -20 °C.

The melting temperatures (T_ms) of the probe–target hybrids are measured in a buffer with a similar composition to that of the hybridization buffer, using the Eco™ Real-Time PCR system (Illumina, San Diego, CA, USA) and SYTO 9 dye (Invitrogen, cat. no. S-34854). The hybridization-like buffer is composed as described in Table 1 and Table 2.

Table 1. Preparation of 5 ml of 35% formamide hybridization-like buffer (for gene detection), part I. Combine the following components. Shake to dissolve DS and incubate at 48 °C until DS is dissolved. Cool to room temperature.

Component	Volume
20x SSC[1]	1.25 ml
DS[2]	0.5 g
0.5 M EDTA, pH 8.0	0.2 ml
MQW[3]	1.3 ml

1 SSC – saline sodium citrate, 2 DS – dextran sulfate sodium salt (Sigma, cat. no. D8906), 3 MQW – MilliQ water; a list of abbreviations is on the last page of this protocol.

Table 2. Preparation of 20 ml of 35% formamide hybridization-like buffer (for gene detection), part II. Add the following components to the mix described in Table 1. Vortex, then spin down. Aliquot and store at -20 °C.

Component	Volume
100% Formamide	1.75 ml
20% SDS[1]	25 ul

1 SDS – sodium dodecyl sulfate

To 1.5 ml of hybridization-like buffer, 3 µl of 5 mM SYTO 9 are added for a final concentration of 10 µM. To 100 µl of the latter mixture, 6 µl of dsDNA (230-350 ng) are added, and the resulting solution is aliquoted into 25 µl portions per well and used for T_m determinations. The T_m needs to be measured for the probe dsDNA (both strands with Dig-dUTP), for target dsDNA (both strands without Dig-UTP) and for a mixture of the probe and the target, which, during the hybridization phase of the thermal protocol, results in hybrid dsDNA (one strand with Dig-UTP and the other without). The thermal protocol used for the T_m determination in hybridization-buffer like buffer is the following: denaturation at 85 °C for 5 min, hybridization at 42 °C for 25 min and melting from 50 °C to 80 °C, at 5.5 °C per sec average ramp rate. Based on the T_m values, the hybridization parameters for the probe mix are determined as detailed in (Moraru *et al.*, 2010; Moraru *et al.*, 2011), which in the present example are: denaturation temperature at 85 °C, hybridization at 42 °C and washing at 42 °C.

GENERAL CONSIDERATIONS

Use autoclaved 0.22 µm filtered MilliQ water during this procedure. Unless stated otherwise, perform incubations at room temperature (RT). All washing steps are carried out in 50 ml volumes, in plastic Petri dishes, when the steps are performed at RT or in the oven, or in 50 ml Falcon tubes, when incubation in a water bath is necessary.

Buffers containing dextran sulfate (DS) are prepared ahead (details see below). First, dissolve the DS at 48 °C (Sigma, cat. no. D8906) in buffer components, with the exception of formamide, SDS and blocking reagents. Second, when the DS is completely dissolved, cool the solution to RT and add the rest of the components. After preparation, formamide-containing buffers are stored at -20 °C, while formamide-free buffers are filtered sterilized (0.2 µm) and kept at 4 °C.

Unless specifically indicated in the protocol, the samples are not allowed to dry at any time during the procedure. Drying is especially dangerous during hybridization or Catalyzed Reporter Deposition (CARD) reactions, when it could cause high background to form. To avoid drying, do all incubations by completely immersing the filters in the respective buffers. When smaller volumes of buffers are used and the incubation is done at a higher temperature and/or for a longer time, the samples need to be placed in humidity chambers. A humidity chamber can be any tightly closed container that seals with a silicone O-ring. For low temperature incubations (e.g., RT, 37 °C, or 46 °C), polypropylene containers can be used. However, for high temperature incubations (e.g., 85 °C), containers made of glass (lid can be of polypropylene) are recommended to avoid deformation of the container and drying of the samples. To create humidity in the chamber, line the bottom with lint-free tissues such as Kimwipes soaked in water or, when the buffers contain formamide or paraformaldehyde, in a formamide-water or paraformaldehyde-water solution of the same concentration as the buffer. For samples immobilized on filters (see section on infected *Pseudoalteromonas* cells), place the filters face-up in Petri dishes, cover them with buffer, and then place the Petri dishes in humidity chambers. For samples directly immobilized on slides (see section on detection of free phage), cover the sample area (marked with a PAP pen, Electron Microscopy Sciences, cat. no. 71310) with buffer and place the slide in a humidity chamber (usually on top of a PCR tube rack).

After being dissolved in water, the horseradish peroxidase (HRP)-labeled oligonucleotides or antibodies are stored at 4 °C for no longer than 6 months. Vortexing should be avoided and mixing is performed by pipetting up and down or gently inverting the tubes.

Prepare $Alexa_{488}$ and $Alexa_{594}$tyramides as described by Pernthaler and Pernthaler (2005). The stocks of fluorochrome-labeled chemicals are stored in the dark. Excessive light exposure during the procedure is to be avoided.

The antibody step promotes the formation of false positives. These are more likely to appear when the cells are damaged during the phageFISH procedure, particularly during the acid treatments (necessary for the inactivation of the HRP, endogenous and introduced with the rRNA targeting probes) and denaturation step. Therefore, the strength of permeabilization, inactivation of peroxidases (by acid treatments or otherwise) and denaturation time must be carefully optimized to minimize damage to cells.

PhageFISH ON INFECTED *PSEUDOALTEROMONAS* CELLS (I)

SAMPLE FIXATION AND IMMOBILIZATION. Fix cells by adding paraformaldehyde (Electron Microscopy Sciences, cat. no. RT 15713) to 500 μ L to 1000 μ L of infected cells to a final concentration of 2%. Shake, but do not vortex the cells. Let incubate for 1 hr at RT. A volume of 100 µl phage lysate is spotted onto PolyLysine glass slides (ThermoScientific, cat. no. J2800AMNZ, pre-cleaned with ethanol and sample area marked with a PAP pen (Electron Microscopy Sciences, cat. no. 71310) and air-dried at 40 °C for 15 min (or until dry). To remove salts, wash 2x PBS 5 min, 1 min in water, followed by 1 min in 96% ethanol and air-drying.

HCl TREATMENTS TO OPEN THE VIRAL CAPSID. Incubate the samples in 0.01 M HCl for 10 min and 0.2 M HCl for 10 min, followed by washings of 1 and 5 min in 1x PBS, 1 min in water, and 4 min in 96% ethanol. Then air-dry. Optionally, can go straight to permeabilization step.

PERMEABILIZATION. For permeabilization, overlay the filters with permeabilization solution (lysozyme buffer, see

Table 3) and 2.0 mg ml^{-1} lysozyme (AppliChem, cat. no. A4972.0010). This solution should be made fresh just prior to using.

Table 3. Twenty ml of permeabilization buffer (enough for 4 slides).

Component	Volume	Final concentration	4 slides
10x PBS 7.4	2 ml	1 x	1 mL
1 M Tris-HCl pH 8.0	2 ml	0.1 M	1 mL
0.5 M EDTA pH 8.0	2 ml	0.05 M	1 mL
MQW	14 ml		7 mL

Incubate for one hour at RT. Then wash for 5 min in 1x PBS and 1 min in water.

INACTIVATION OF ENDOGENOUS PEROXIDASES. For inactivation, immerse the filters in 0.01M HCl for 10 min, followed by washing in 1x PBS for 5 min, in water for 1 min and in 96% ethanol for 1 min, followed by air-drying for 3-5 min.

rRNA HYBRIDIZATION. As an example, hybridization with probe EUB338 (Amann *et al.*, 1990) is described. Add the HRP-labeled probe EUB338 (Biomers, Ulm, Germany) to a final concetration of 0.16 ng μl^{-1} to the hybridization buffer containing 35% formamide (for details see Table 4 and Table 5. Mix by gentle shaking, no vortexing, to avoid the removal of the HRP from the oligonucleootide. Then cover the filter pieces with hybridization mixture and place them in a humid (35% formamide solution) chamber. Hybridize O/N at 46 °C. On the following day, wash for 15 min at 48 °C in prewarmed washing buffer (for details see Table 6).

Table 4. Twenty ml of 35%-formamide hybridization buffer for rRNA detection, part I. Dissolve DS in a water bath of 48 °C to 60 °C. Cool down to RT, then proceed with Table 5.

Component	Volume	final concentration
5 M NaCl	3.6 ml	0.9 M
1 M Tris-HCl	0.4 ml	20 mM
MQW	7 ml	
DS	2 g	10%

Table 5. 35%-formamide hybridization buffer, part II. Vortex and heat at 46 °C

Component	Volume	final concentration
20% SDS	0.02 ml	0.02%
10% BR[1]	2 ml	1%
sss DNA [10 mg ml^{-1}] [2]	0.5 ml	0.25 mg ml^{-1}
Yeast RNA [10 mg ml^{-1}] [3]	0.5 ml	0.25 mg ml^{-1}
Formamide	7 ml	35%

1 BR – Blocking Reagent (Roche, Germany, cat. no. 11096176001), 2 sss DNA – sheared salmon sperm DNA (Ambion, cat. no. AM 9680), 3 Ambion, cat. no. AM 7118

Table 6. Fifty ml washing buffer (enough for 2 slides) for 35% formamide hybridization buffer and 48°C washing.

Component	Volume	final concentration
5 M NaCl	700 µl	70 mM
1 M Tris-HCl pH 8.0	1 ml	20 mM
0.5 M EDTA pH 8.0	0.5 m	5 mM
MQW	*ad* 50 ml	
20% SDS	25 µl	0.1%

CARD FOR rRNA DETECTION. Equilibrate the samples for 15 min in 1x PBS. Then incubate the samples for 30 min at 37 °C in amplification buffer combined just prior to use with 0.0015% H_2O_2 and aliquoted 0.33µg ml^{-1} $Alexa_{488}$-labeled tyramides. **At this point all slide work should be done in the dark**.

Table 7. Forty ml amplification buffer for rRNA detection at 10% DS, part I. Combine the following components and dissolve DS at 48°C to 60°C in a water bath. Cool down to RT and proceed with Table 8.

Component	Volume	final concentration
10x PBS	4 ml	1x
5 M NaCl	16 ml	2 M
MQW	*ad* 35 ml	
DS	4 g	10%

Table 8. Amplification buffer for rRNA detection at 10% DS, part II. Add the following components to the mix of Table 7. Sterile-filter through 0.2 um, aliquot and store at 4°C.

Component	Volume	final concentration
10% BR	0.4 ml	0.1%
MQW	*ad* 40 ml	

Wash for 10 min with 1x PBS at 46 °C, then for 1 min in water at RT and finally, for 1 min in 96% ethanol, followed by air-drying.

RNase TREATMENT. Overlay samples with RNase solution (0.5 U $µl^{-1}$ RNase I [Ambion, cat. no. AM 2295], 30 µg ml^{-1} RNase A [Sigma, cat. no. R4642-10], 0.1 M Tris-HCl, pH 8.0) (for details see Table 9) and incubate 3-4 hr at 37 °C. Alternatively, can incubate O/N.

Table 9. Approximately 10 mL RNase solution (enough for >12 slides).

component	Volume	Final Concentration	Volume (4 slides)
1 M Tris HCl, pH 8.0	1 ml	99.4 mM	300 μ L
MQW	9 ml		3 mL
100 U/µl RNase I	50 µl	0.497 U/µl	17.5 μ L (15-20)

30 mg/ml RNase A	10 µl	29.8 mg/ml = 29.8 µg/µl	3 μ L
total	10.06 ml		

Afterwards, wash 5 min in 1x PBS and then 1 min in water.

INACTIVATION OF HRP INTRODUCED WITH THE rRNA PROBE. For the inactivation of HRP, incubate the samples for 10 min in 0.2 M HCl, wash in 1x PBS for 5 min, then 1 min with water, 1 min with 96% ethanol. Let air-dry. Ensure the slides are protected from light.

GENE HYBRIDIZATION. For prehybridization overlay samples with the same buffer as for hybridization, but without the probe, for 30 min at 42°C (the same temperature as hybridization). The composition of the hybridization buffer is described in Table 10 and Table 11.

Table 10. Ten ml of 35%-formamide hybridization buffer for gene detection, part I. Dissolve DS in a water bath of 48C to 60C. Cool down to RT, then proceed with Table 11.

component	Volume	Final Concentration
20x SSC	2.5 ml	5x
0.5 M EDTA, pH 8.0	0.4 ml	20 mM
MQW	1.1 ml	
DS	1 g	10%

Table 11. 35%-formamide hybridization buffer for gene detection, part II. Add the following components to the mix of Table 10. Then aliquot and store at -20°C.

component	Volume	Final Concentration
20% SDS	0.05 ml	0.1%
10% BR	1 ml	1%
sss DNA [10 mg ml^{-1}]	0.25 ml	0.25 mg ml^{-1}
Yeast RNA [10 mg ml^{-1}]	0.25 ml	0.25 mg ml^{-1}
Formamide	3.5 ml	35%

For hybridization, the samples are transferred into probe-containing hybridization buffer. At 23 min (into the 30 min incubation, above), add probes to 5 mL of hybridization buffer. Samples are hybridized with 6 probes (in the present example probes P*runk1-6*). Each probe is added to a final concentration of 6 pg µl^{-1}. As negative control, an extra set of samples is combined with hybridization buffer containing the nonsense probe Non-Poly350Pr (Moraru *et al*. 2010) at a final concentration of 30 pg µl^{-1} (the equivalent of 6 *unk* probes). The samples are first denatured for 45 to 50 min (never beyond 1 hr) depending on the sample (needs to be optimized for individual experiment) in a hybridization oven at 85 °C. After denaturation, immediately transfer the samples to a 42 °C oven and hybridize for 18–22 h (i.e. O/N).

Remember to set up humidity chambers for a) pre-hybridization as well as for b) denaturation and c) hybridization (see General considerations). Next, perform the following washes: first, 3x

washing buffer I (WBI, see Table 12) for 1 min at RT in a petri dish, then WBI for 30 min at 42 °C in a falcon tube, followed by washing buffer II (WBII, see Table 13) 3x for 1 min at RT and 1.5 h at 42 °C in a slow shaking water bath, and finally 1 min with 1x PBS at RT (alternatively, 42 C).

Table 12. Fifty ml of washing buffer I (WBI) for gene detection (enough for 2 slides). This will go into a 50 mL falcon tube and remainder to a petri dish.

Component	Volume	Final Concentration	Na^+ contribution (mM)	
20x SSC	5 ml	2x	390	20
MQW	*ad* 50 ml			200
20% SDS	250 µl	0.1%	3.45	1 mL
total	**50 ml**		**393.45**	

Table 13. Fifty ml of washing buffer II (WBII) for gene detection.

Component	Volume	Final Concentration	Na^+ contribution (mM)
20x SSC	250 µl	0.1x	19.5
MQW	up to 50 ml		
20% SDS	250 µl	0.1%	3.45
total	**50 ml**		**22.95**

ANTIBODY BINDING. Incubate the samples in antibody blocking solution (see Table 14) for 45 min at 37 °C in a water bath, *without shaking*. Transfer slides from 1 falcon to another falcon tube with Table 14.

Table 14. Fifty ml of antibody blocking/washing solution (enough for 2 slides).

Component	Volume	Final Concentration
10x PBS, pH 7.4	5 ml	1x
10% Western Blocking Reagent[1]	5 ml	1 %
MQW	*ad* 50 ml	

1 WBR, Roche, cat. no. 11921673001

For antibody binding, incubate the samples in antibody buffer combined with 0.3 U ml^{-1} (500x dilution of the 150 U/ml stock, or 25 μ L into 10 mL) anti-Dig HRP-conjugated antibody (Fab fragments; Roche, cat. no. 11207733910) for 1.5 h.

Table 15. Ten ml antibody buffer.

Component	Volume	Final Concentration
10x PBS pH 7.4	1 ml	1x
10% WBR	1 ml	1 %

MQW	8 ml

Wash samples in the used antibody blocking/washing buffer 1x at 37 C for 1 min, then 20x PBS/Tween 3x 10 min. All steps are carried out in a water bath at 20 rpm.

CARD FOR GENE DETECTION. The samples are overlaid with amplification buffer containing 1x PBS, 20% dextran sulfate, 0.1% blocking reagent, and 2 M NaCl with 0.0015% H_2O_2 and 2 µg ml^{-1} $Alexa_{594}$-labeled tyramide and incubated for 45 min at 37 °C in oven. Then wash 2x 10 min in 1x PBS in a 46 °C water bath, slow shaking, then 1 min in water, 1 min in 96% ethanol (petri dish), followed by air-drying.

Table 16. Forty ml amplification buffer (enough for 16 slides) for rRNA detection at 20% DS, part I. Combine the following components and dissolve DS at 48°C to 60°C in a water bath. Cool down to RT and proceed with Table 17.

component	volume	final concentration
10x PBS	4 ml	1x
5 M NaCl	16 ml	2 M
MQW	*ad* 35 ml	
DS	8 g	20%

Table 17. Amplification buffer for rRNA detection at 20% DS, part II. Add the following components to the mix of Table 16. Sterile-filter through 0.2 um, aliquot and store at 4°C.

component	volume	final concentration
10% BR	0.4 ml	0.1%
MQW	*ad* 40 ml	

EMBEDDING AND COUNTERSTAINING. The filters are embedded in either ProLong Gold antifade reagent (Invitrogen, cat. no. P36930) or SlowFade Gold antifade reagent (Invitrogen, cat. no. S36936) containing 1 µg ml^{-1}4´,6-diamidino-2-phenylindole (DAPI) by adding 4-5 µL "drop" to center of sample and stored at -20 °C.

PhageFISH ON PHAGE LYSATES (FREE PHAGE PARTICLES) (II)

MICROSCOPY, CELL COUNTS AND IMAGE PROCESSING OF phageFISH SAMPLES

MICROSCOPY AND IMAGE ACQUISITION. For microscopy use an epifluorescence microscope, e.g., Axioskop2 Mot Plus (Carl Zeiss, Germany), equipped with the following fluorescence filter sets: $Alexa_{488}$ (472/30 excitation, 520/35 emission, 495 Beam Splitter) and $Alexa_{594}$ (562/40 excitation, 624/40 emission, 593 Beam Splitter). The $Alexa_{488}$ filter set is used for detection of the 16S rRNA signals (green), while the $Alexa_{594}$ filter set is used for detection of the phage gene signals (red). Both for cell counts and image processing, take photomicrographs with a black and white digital camera, e.g., AxioCamMn (Carl Zeiss, Germany), using the AxioVision 4.8 software (Carl Zeiss, Germany). To capture both the strong, cell-wide and the weak, dot-like phage signals, take a series of images with increasing exposure times (e.g. 3 ms, 5 ms, 7 ms, 10 ms, 15 ms, 25 ms, 40 ms, 50 ms, 75 ms, 100 ms and 140 ms) for the $Alexa_{594}$ filter set. Then have the black and white photomicrographs pseudo-colored automatically by the software used for acquisition, green for the 16S rRNA and red for the phage signals.

COUNTING. Count signals on photomicrographs, by manually marking the cells in the $Alexa_{488}$ channel and the corresponding gene signals in the $Alexa_{594}$ channel with the “Events” tool from the “Measure” menu. The number of events is determined using the “measure events” function. Count at least 800 cells per sample. Always correct the number of infected cells for the number of false positives found in the negative control (infected cells hybridized with the NonPoly350Pr probe).

IMAGE PROCESSING. The processing of the images of phageFISH preparations is divided into two stages: (i) exporting selected fields of view using the Zen Lite 2011 software (Blue edition; Carl Zeiss, Germany), and (ii) reconstructing composite images from the original images of the exposure time series using the PaintShop Photo Pro X4 software (Corel Corporation, USA).

More specifically, in the event that the signals in the $Alexa_{594}$ filter set are characterized by different sizes and intensities in such a way that the exposure times at which the large signals are not overexposed did not allow the small signals to be visible, while the exposure times at which the small signals were visible resulted in a serious overexposure of the large signals, some image processing, i.e., image reconstruction from several images, is recommended in order to achieve a representative image for documentation. To reconstruct the image, devise a High Dynamic Range Imaging protocol. Accordingly, images with increasing exposure times are loaded as separate layers. First, different elements composing an image are identified. Then, for each element, the layer where the element was clearly visible, but not overexposed, is selected. The elements are merged into one new image, by transferring the information from the higher exposure layers to the lower exposure layers, using the “Eraser” tool. At the end, a sharpening filter and a black threshold are applied on the reconstructed images. The overlay between the green 16S rRNA signals and the red phage signals is generated by visualizing the layers with the “Lighten” function.

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ABBREVIATIONS

ad	fill up to indicated volume
BR	Blocking Reagent
CARD	Catalyzed reporter deposition
Dig	Digoxigenin
DS	Dextran sulfate
HRP	Horseradish peroxidase
MQW	MilliQ water
SDS	Sodium dodecyl sulfate
SSC	Saline sodium citrate
sss DNA	Single-stranded salmon DNA
WB	Washing buffer
WBR	Western Blocking Reagent

REAGENTS AND BUFFERS

Reagent	Preparation
0.2 M HCl	Dilute 1 M HCl (ordered) in MQW
0.01 M HCl	Diluted from 0.2 M HCl
SDS	(g/mL) Use autoclaved MQW, then filter sterilize. Not stable beyond 3-6 months
PBS	10 tablets / 1 L MQW, autoclave, then filter sterilize
0.5 M EDTA	
5 M NaCl	