phageFISH Protocol

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Abstract

This protocol combines phage gene detection with rRNA detection for the identification of host cells and detection of free phage particles.

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Guidelines

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)-abeled 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 Alexa488 and Alexa594tyramides as described by Pernthaler and Pernthaler (2005). The stocks of fluorochrome-abeled 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 Psuedoalteromonas cells (I):

SAMPLE FIXATION. Fix cells by adding paraformaldehyde (Electron Microscopy Sciences, cat. no. RT 15713) to a final concentration of 2% and incubate overnight at 4 °C.

SAMPLE IMMOBILIZATION. After paraformaldehyde fixation, filter the cells onto 0.2 µm polycarbonate filters (GTTP, Millipore, cat. no. GTTP02500). Then wash in 15 ml water, air-dry and store at -20 °C.

PERMEABILIZATION. For permeabilization overlay the filters with permeabilization solution which is made of lysozyme buffer (see Table 3) and 0.5 mg ml-1 lysozyme (AppliChem, cat. no. A4972.0010). This solution should be made fresh just prior to using.

Table 3: lysozyme buffer (20 ml).

Component	Volume	Final Concentration
10x PBS 7.4	2ml	1x
1M Tris--HCl pH 8.0	2ml	0.1M
0.5M EDTA pH 8.0	2ml	0.05M
MQW	14ml	

Incubate for one hour on ice. 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.

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 for 3 h at 46 °C, and then 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 48C to 60C. Cool down to RT, then proceed with Table 5.

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

Table 5. 35%---formamide hybridization buffer, part II.

Component	Volume	Final Concentration
20% SDS	0.02ml	0.02%
10% BR1	2ml	1%
sss DNA [10mg ml-1]2	0.5ml	0.25 mg ml-1
Yeast RNA [10 mg ml-1]3	0.5ml	0.25 mg ml-1
Formamide	7ml	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 for 35% formamide hybridization buffer and 48°C washing.

Component	Volume	Final Concentration
5 M NaCl	700µl	70 mM
1 M Tris--HCl pH 8.0	1 ml	20 mM
0.5 M EDTA ph 8.0	0.5 ml	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 10 min at 37 °C in amplification buffer combined just prior to use with 0.0015% H2O2 and 0.33µg ml-1 Alexa488 labeled tyramides.

Table 7. Forty ml amplification buffer for rRNA detection at 10% DS, part 1. 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	2M
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 0.2µm, aliquot and store at 4°C.

Component	Volume	Final Concentration
10% BR	0.4ml	0.1%
MQW	ad 40ml	

Wash for 10 min with 1x PBS at 46 °C, then for 1 min in water 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 overnight at 37 °C.

Table 9. RNase solution.

Component	Volume	Final Concentration
1 M Tris HCl, pH 8.0	1 ml	99.4 mM
MQW	9ml	
100 U/µl RNase I	50µl	0.497 U/µl
30 mg/ml RNase A	10µl	29.8 mg/ml + 29.8µg/µl
total	10.06 ml	

Afterwards, wash 2x 5 minutes 1x PBS and then 1 minute 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, then wash in 1x PBS for 1 and 5 min, then 1 min with water, 1 min with 96% ethanol. Let air dry.

GENE HYBRIDIZATION. For prehybridization overlay samples with the same buffer as for hybridization, but without the probe, for 3.5 h at 42°C. 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	2.5ml	5x
0.5 M EDTA, pH 8.0	0.4ml	20 mM
MQW	1.1ml	
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	0.05ml	0.1%
10% BR	1ml	1%
sss DNA [10 mg ml-1]	0.25 ml	0.25 mg ml-1
Yeast RNA [10 mg ml-1]	0.25ml	0.25 mg ml-1
Formamide	3.5ml	35%

For hybridization, the samples are transferred into probe containing hybridization buffer. Samples are hybridized with 6 probes (in the present example probes Prunk1-6). Each probe is added to a final concentration of 5 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 – 35 min to an 1 hour 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. 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, then WBI for 30 min at 42 °C, 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.

Table 12. Fifty ml of washing buffer I (WBI) for gene detection.

Component	Volume	Final Concentration	Na+ contribution (mM)
20x SSC	5 ml	2x	390
MQW	ad 50ml	0.1%	
20% SDS	250 µl		3.45
Total	50ml		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	50ml		22.95

ANTIBODY BINDING. Incubate the samples in antibody blocking solution (see Table 14) for 45 minutes.

Table 14. Fifty ml of antibody blocking/washing solution.

Component	Final Volume	Final Concentration
10x PBS, pH 7.4	5 ml	1x
10% Western Blocking Reagent1	5 ml	1%
MQW	ad 50ml	

1 WBR, Roche, cat. no. 11921673001For antibody binding, incubate the samples in antibody buffer combined with 0.3 U ml-1 (500x dilution of the 150 U/ml stock) anti Dig HRP conjugated antibody (Fab fragments; Roche, cat. no. 11207733910) for 1.5 h.

Table 15. Ten ml antibody buffer.

Components	f.v.=10 ml	f.c.
10x PBS pH 7.4	1 ml	1x
10% WBR	1 ml	1%
MQW	8 ml	

Then wash samples in a solution of 1x PBS and 1% WBR (see Table 14) solution for 1 min and 3x 10 min. All steps are carried out on a shaker 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% H2O2 and 2 µg ml--1 Alexa594 labeled tyramide and incubated for 45 min at 37 °C. Then wash for 1 min, 5 min and 2x 10 min in 1x PBS in a 46 °C oven, slow shaking, then 1 min in water, 1 min in 96% ethanol, followed by air drying.

Table 16. Forty ml amplification buffer 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	0.4ml	0.1%
MQW	ad 40ml	

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-14´,6-diamidino-2-phenylindole (DAPI) and stored at -20 °C.

phageFISH on Phage Lysates (Free Phage Particles) (II):

SAMPLE IMMOBILIZATION. 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 37 °C for 50 min. To remove salts, wash 1 min in water, followed by 1 min in 96% ethanol and air drying.

FIXATION. Overlay the sample area with a 1% PFA solution and incubate for 1 hour at room temperature. Then wash for 5 min in 1x PBS, 1 min in water, and 1 min in 96% ethanol, followed by 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.

GENE DETECTION. From here on, follow the protocol above, starting with gene hybridization.

EMBEDDING AND COUNTERSTAINING. Cover the marked sample area with SYBR Green-containing mounting media (Lunau et al., 2005)

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: Alexa488 (472/30 excitation, 520/35 emission, 495 Beam Splitter) and Alexa594 (562/40 excitation, 624/40 emission, 593 Beam Splitter). The Alexa488 filter set is used for detection of the 16S rRNA signals, while the Alexa594 filter set is used for detection of the phage gene signals. 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 Alexa594 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 Alexa488 channel and the corresponding gene signals in the Alexa594 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 Alexa594 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.

References:

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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

Before start

Before staring 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 (= 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 2.5−3% agarose gels, for size and Dig incorporation. The Dig--labelled probes position themselves at a higher position in the gel than the respective targets, a clear indication of Dig incorporation. The concentration of PCR products (probes and targets) is best determined using a Quant--iT™Picogreen assay (Invitrogen, cat. no. P7589) or spectrophotometrically, using a NanoDrop instrument. The probes were stored at --20 °C.

The conditions for hybridization need to be determined as described by Moraru and colleagues (2010). Firstly, the melting temperature (T_m) for each probe−target pair is calculated using the PolyPro software (Moraru *et al.*, 2011). Secondly, 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]	1.25ml
DS[2]	0.5g
0.5M EDTA, pH 8.9	0.2ml
MQW[3]	1.3ml

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	1.75ml
20% SDS[1]	25µl

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.

Protocol