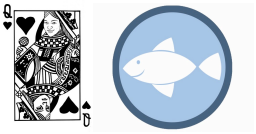
CARD-FISH and Microautoradiography Protocol for Bacteria and Archaea



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CARD FISH Hybridization

Sample Fixation

1. Fix samples with paraformaldehyde or 35% formaldehyde to final concentration of 2-4% and fix for at least 1 but not longer than 18h.
2. Filtrate sample (for ~10^6 cells/ml 10ml will do) on white polycarbonate (25 mm 0.2 μm) filter with cellulose nitrate support filter (0.45 μm).
3. After sample filtration, wash with 5-10 ml of MQ
4. Air-dry filters
5. Store at –20ºC until processing. Filters can be stored frozen for several months.

Embedding

1. Warm up 0.1% Agarose and pipette 30μl on petri dish. Dip filter with both sides in Agarose and place filters shiny side down on drop
2. Let filters dry at 37ºC in hybr. oven without lid for 10-15min.
3. Pour ethanol (95%) in petri dish and carefully remove filters
4. Dry filters, now they can be stored at -20°C

Permeabilization

1. Prepare 10ml of permeabilization mix with **either** lysozyme (Eub) **or** proteinase K (Arch); after preparation put on ice

Stock reagent	Volume (µl)	Final
Lysozyme	100mg	10mg/ml
Proteinase K	2check	10.9mg/ml
1M Tris-HCl	1000	0.1M
0.5M EDTA	1000	0.05M
MQ water	8000	

2. Pour lysozyme (proteinaseK) in petri dish and place filters upside down into it, incubate for 1h at 37ºC in hybr. oven
3. After 1h wash filters in MQ (1x for Lysozyme, 3x for Proteinase K)
4. Place filters for 20-25min. in 0.01M HCL at RT
5. Wash filters 2x in excess MQ
6. Shortly dip them into Ethanol (95%)
7. Dry filters, now they can be stored at -20.

Hybridization

1. Prepare hybridization buffer:
2. Cut 25mm filters in up to 8 sections and place them into 0.7ml Eppi (labeled).
3. Mix 300μl hybridization buffer + 15μl HRP probe. Probe to buffer ratio: 1:20. Freeze probes just once, after thawing store them at 4ºC for up 0.5 years.
4. Hybridize at 35ºC for 12h – 15h (low activity), stick Eppis around rotor, darken them and shake slowly
5. Prepare washing buffer:

Washing Buffer for 35ºC and 55% Formamide in Hybridization buffer:

Stock reagent	Volume (µl)	Final
5M NaCl	30*	13mM
1M Tris-HCl	1000	20mM
0.5M EDTA	500	5mM
MQ water	48420	
10% SDS	50	0.01%

Washing Buffer for 35ºC and 20% Formamide in Hybridization buffer:

Stock reagent	Volume (µl)	Final
5M NaCl Arch	1350*	145mM
1M Tris-HCl	1000	20mM
0.5M EDTA	500	5mM
MQ water	47100	
10% SDS	50	0.01%

1. Pre warm buffer at 37ºC.
2. After hybridization quickly transfer filter sections 15 min in washing buffer, afterwards pour in to buechner funnel
3. *chemical corrections applied (mM -100μL)

Amplification

1. Prepare PBS-T-Mix:

Stock reagent	Volume (µl)	Final
1xPBS	49750	
100% Triton X100	25	0.05%

PBS should have pH of 7.4 -7.6 (better peroxidase turnover)

1. Pick filters and incubate in PBS-T-Mix at RT for 10-15min.
2. Prepare substrate mix with prepared amplification buffer (AMP) and 30% H2O2
3. Dilute 30% H2O2 to final concentration of 0.0015% (Step A to B; all Volumes in μl)

	AMP	H2O2	Tyr	Ratio	
A	200	1 from 30%			
B	493	5 from A	2.5	1:200	A555
B	493	5 from A	3.4	1:150	A488
B	493	5 from A	5.0	1:100	

1. Dab filters on blotting paper, place sections on top of each other and place in 1.5ml Eppi
2. Incubate in substrate mix for 10min up to 45min. at 37ºC in hybrid. oven in the dark
3. After incubation in Substrate mix dab filters on blotting paper
4. Wash in 50ml PBS-T mix at RT for 10min in the dark
5. Wash in MQ
6. Wash in Ethanol 95%
7. Dry and mount in DAPI mix

Hybridization Buffers - 10ml:

Store at -20ºC for up to 1 year; if in use keep on ice !

Buffer with 55% Formamide:

for EUB, ALF, BET, GAM, CF

Stock reagent	Volume (µl)	Final conc.
Dextran Sulfate	1g	10%
5M NaCl	1800	900mM
1M Tris-HCl	200	20mM
100% Triton X100	5	0.05%
Bring in solution at 40-60ºC in water bath, takes about 30min then cool down on ice. Then add:		
Formamide	5500	55%
10% Blocking	1000	1%
Sigma Water	1500	

Buffer with 20% Formamide:

for CREN and EUR

Stock reagent	Volume (µl)	Final conc.
Dextran Sulfate	1g	10%
5M NaCl	1800	900mM
1M Tris-HCl	200	20mM
100% Triton X100	5	0.05%
Bring in solution at 40-60ºC in water bath, takes about 30min then cool down on ice. Then add:		
Formamide	2000	20%
10% Blocking	1000	1%
Sigma Water	5000	

Amplification Buffer - 20ml:

Store at +4°C

Stock reagent	Volume (µl)	Final conc.
Dextran Sulfate	2g	10%
5M NaCl	8000	2M
10% Blocking	200	0.1%
1x PBS	11800	

DAPI mix - 2ml

Stock reagent	Volume (µl)	Final
DAPI 50µg/ml	40	1 µg/ml
1xPBS	140	0.5
Vectashield	280	1
Cititfluor	1540	5.5

DAPI mix - 5ml

Stock reagent	Volume (µl)	Final
DAPI 50µg/ml	200	2 µg/ml
1xPBS	350	0.5
Vectashield	700	1
Cititfluor	3750	5.5

Blocking Reagent 10% - 100ml

Prepare Maleic acid buffer

1. 100mM Maleic acid
   MW: 116.08g/mol
   11.608g/l
2. 150mM NaCl
   MW: 58.44g/mol
   8.766g/l
3. adjust pH with a lot of solid NaOH to 7.5
4. Add Boehringer Mannheim Blocking reagent to a final concentration of 10%
   10g with 100ml Maleic Acid buffer
5. Dissolve reagent on heating plate at around 60ºC with stirring for about 1h, do not boil
6. autoclave solution
7. Aliquot to 10ml parts and store them frozen at -20ºC; According to the manufacture solution should be good for a few days at 4ºC and many month if stored frozen.

Paraformaldehyde 20%

1. Turn on a 60 °C water bath in advance.
2. Pour 150 mL of milliQ water into a 250 mL screw cap polycarbonate bottle. Add 50 g of paraformaldehyde.
3. Fasten the cap securely and shake well to disperse the paraformaldehyde powder.
4. Add 3 mL of 1 N, NaOH.
5. Warm to 60 °C for 20 to 25 minutes shaking the bottle ever 5 minutes. Most but not all of the paraformaldehyde will dissolve in this time. It will still be quite cloudy. Don't worry. The last step is 0.2 µm filtration that will clean it up nicely.
6. Add 3 mL of 1 N HCl
7. Add 25 mL of 100 mM sodium phosphate buffer, pH 7.2.
8. Add 25 mL of 5 M NaCl.
9. Adjust pH to 7 using pH paper. This will take approximately 400 µL of 1N, NaOH. If you cook too long at 60°C it will be very acidic making it difficult to adjust the pH.
10. Adjust the final volume to 250 mL with Milli Q using the graduations on the polycarbonate bottle.
11. Filter using a 0.22 µm Millipore type GS filter (47 mm) using a designated formaldehyde side arm flask, frit and cup. There may be a substantial layer of paraformaldehyde on the filter.
12. Transfer the filtrate to a clean 250 mL polycarbonate bottle. Label with the date concentration (20% formaldehyde) and your initials.
13. Store at 4°C. Fresh formaldehyhde is good for 2 or days if kept in the refrigerator.

Phosphate buffers for PBS

10x PBS pH 7.7

2.76g NaH2PO4
14.24g Na2HPO4
75.97g NaCl
fill up to 1L with MQ

EDTA DisoDihy 0.5M

MW: 372.24g/mol
186.12g/l
add NaOH pastilles, only at pH 8 EDTA will dissolve!
fill up to 1 liter with MQ
pH 8.0; autoclave

SDS 10%

50g to 500ml MQ
do not autoclave!
dissolves at 40ºC in water bath

Tris-HCL 1M

MW: 121.14g/mol
157.6g/l
With 6M HCL
pH 8.0
up to 1 Liter MQ

NaCL 5M

MW: 58.44g/mol
146.1g/500ml
autoclave

Abbreviations used

DMF: N,N-Dimethylformamide
F_W=73.09g/mol
TEA: Triethylamine
F_W=101.19g/mol
TYR-HCL: Tyramine-HCL
M_w = 173.64g/mol

Solutions

1. **DMF-TEA stock:**
   1ml DMF + 10µl TEA
   Prepare in 2ml Eppi
2. **TYR Stock:**
   10mg TYR-HCL + 1ml DMF-TEA stock
   100µl TYR.stock = 5.76µmol/l
   Prepare in 15ml Greyner

Triethylamine & Dimethylformamide are dangerous: work in the hood and on ice!

3. **Succinimidyl ester:**
   1mg active ester + 100µl DMF
   1 mg $Alexa_{488}$ = 1.6 µmol/l

   $Alexa_{488}$: M_w = 643.41g/mol
   λ_{max} = 495nm
   E_{max} = 519nm
   ε = 71,000
   C_f = 0.11

   $Alexa_{555}$: M_w = 1250g/mol
   λ_{max} = 555nm
   E_{max} = 565nm
   ε = 150,000
   CF_{280} = 0.08

Esters are light sensitive and prone to hydrolysis, therefore prepare shortly before tyramide synthesis; cool esters on ice until they are used for synthesis.

Synthesis

1. 100µl $Alexa_{488}$ + 25.2µl TYR stock
   100µl $Alexa_{555}$ + 12.6µl TYR stock
2. Incubate at room temperature in the dark for ~12 hours, slowly rotating
3. Dilute to 1ml with absolute Ethanol (874.8µl for A_{488} and 887.4µl for A_{555})
4. Make aliquots (~50µl) and store at -20°C.

Chemicals needed

1mg A_{488} Succinimidyl ester:
Molecular Probes Cat.Nr. A-20000

1mg A_{555} Succinimidyl ester
Molecular Probes Cat.Nr. A-20009

N,N-Dimethylformamide (waterfree)
Sigma D 4551

Tyramine-HCL
Sigma T2879

Triethylamine
Sigma 17924

Microautoradiography

Cell sampling and fixing

1. Check the abundance of bacteria in your samples, to estimate the volume to filter (see step 6) and the amount of substrate to add. For example:

Abundance of bacteria in sample	Volume to filter (ml)
$1x10^4$ cell ml^{-1}	50 (minimum)
$5x10^5$ cell ml^{-1}	10
$1x10^6$ cell ml^{-1}	5

2. Transfer samples in greyner tubes. 2 samples and 1 control. Kill control with PFA (final conc. ~2%) and wait for 15min.
3. Add radioactive labeled compounds, e.g.:

Substrate	Final concentration in sample*
^{3}H-Glucose	20 nM
^{3}H-Leucine	20 nM
^{3}H-Thymidine	20 nM
^{14}C-HCO_3^-	100µCi

*Above concentrations are for marine systems

4. Incubate for at least 4h in the dark under in situ temperature. Take into account the expected level of activity for your sample and the substrate activity (e.g. for deep sea 24h for ^{3}H-leucine, 72h for ^{14}C-bicarbonate).
5. Kill all samples with PFA (final conc. ~2%) and incubate samples at 4ºC in the dark for about 18h.
6. Filter the samples on 25mm 0.2μm GTTP filters using a Manyfold and nitrocellulose support filters
7. Wash the filters 2x with 5ml fresh MQ.

Transfer of filter sections

All the following steps must be performed in the dark with a safety lamp, although complete darkness ensures a better result!

1. Melt photographic emulsion for 15min at 43ºC in a water bath in the dark.
2. Coat the slides with the emulsion (see preparation notes) by dipping them into the tube (2 slide mailer).
3. Wipe away the emulsion from the underside of the slide with clean tissue paper.
4. Dry the slides for 5-10 min. (the time it takes to process 4 slides) on ice cold aluminum plate. This is best done in a small tray containing ice covered with aluminum foil.
5. One by one, take the prepared filter sections which are in nice order and carefully mount them on the slides, cells facing to the emulsion.
6. Once each slide is mounted, return it to the ice-tray, to be briefly dry, while processing the others
7. Place the slides in light proof box wrapped with aluminum foil, containing silica gel drying beads, for exposure at 2- 4ºC. The exposure time is dependent on the activity of the substrate and expected activity of the cells. See appendix for table of suggested guidelines.

*For substrates not included in the table an appropriate exposure time should be tested.

Developing and Fixing

1. Place box with slides in the dark room (17°C) for one hour before starting.
2. Prepare developing chemicals in slide staining jars

All the following steps must be performed in complete darkness!

3. Remove aluminium foil and place the slides in the slide rack.
4. Develop the slides using Kodak specifications: 2 min in Dektol Developer, 10s to stop developing in MQ, 5 min in fixer, 2 min in MQ.

*The Dark room should be at around 17ºC, especially the developer and fixer. All developing chemicals and MQ should be kept at this room temperature.

After this you can turn on the light!

1. Keep the slides in the MQ until you are back in the lab. Dry the underside of the slide
2. Surround the borders of the filters on the underside of the slide with a marker. This is also the last chance to know from the filter labels which section is which - so label the slide accordingly.
3. Dry a bit with paper the side with filters, but not too much, otherwise it is difficult to remove the filters. If the filter is too dry and sticks to the slide, it can be rewet with a drop of water.
4. Carefully peel the filter sections off
5. Put a drop of DAPI-mix in each filter outline and put a cover slip over it.
6. They can be stored at -20ºC until they are counted on the epifluorescence microscope.

Preparing the emulsion:

The Emulsion is a mix of NTB and Sigma water 2:1 (v/v) = 7ml + 3.5ml

1. Melt the emulsion for 45-60min at 43-45°C before use.
2. Wrap the 2 slide mailer (tubes) with colored PVC tape and ensure that the lid is covered with black marker pen.
3. Add 3.5 ml Sigma water.
4. In the dark room, add 7ml of photographic emulsion.

The emulsion is highly sensitive to light, therefore, complete darkness is recommended. This is best done using a bottletop dispenser (e.g. Dispensette, Sigma-Aldrich).

5. Wrap the tubes in aluminum foil and store at 4°C.

*Take into account the emulsion has an expiration date of few months. Each tube with the emulsion can be re-used a maximum of three times.

Suggested exposure time for various substrates according to literature

Substrate	Concentration (nM)	Emulsion	Incubation Time (h)	Temperature (°C)	Location	Reference
^{3}H-AA mixture (47 Ci/mmol^{-1})	2.1	NTB-2	48	-20	Delaware Bay	Cottrell and Kirchman 2000
^{3}H-NAG (9,9 Ci/mmol^{-1})	10	NTB-2	48	-20		
^{3}H-Protein [from Vibrio grown on ^{3}H-leucine]		NTB-2	48	-20		
^{3}H-Chitin [from fungus grown on ^{3}H-NAG]		NTB-2	48	-20		
^{3}H-Thymidine (83,5 Ci mmol^{-1})	20	NTB-2	48	4	Delaware estuary	Cottrell and Kirchman 2003
^{3}H-Leucine (150 Ci mmol^{-1})	20	NTB-2	48	4		
^{3}H-AA mixture (47 Ci mmol^{-1})	0.5	NTB-2	48-144	4	Noth Atlantic Ocean	Malmstrom et al. 2004
^{35}S-DMSP (1,170 Ci mmol^{-1})	<0.1	NTB-2	336-480	4		
^{3}H-Thymidine (83,5 Ci mmol^{-1})	20	NTB-2	28-48	4	Delaware estuary	Cottrell and Kirchman 2004
^{3}H-Leucine (150 Ci mmol^{-1})	20	NTB-2	3-7	room T		
^{35}S-DMSP (12-43 TBq mmol^{-1})	<0.1	LM-1	48-144	4	Noth Atlantic Ocean	Malmstrom et al. 2004
^{3}H-Protein (0,3-0,9 Ci/gr^{-1}) [from *Vibrio* grown on ^{3}H-Leucine]	20 ng/ml	NTB-2	10 days	4	Noth Atlantic Ocean	Malmstrom et al. 2005
^{3}H-AA mixture (47 Ci/mmol^{-1})	0.5	NTB-2	48-72	4		
^{3}H-Glucose (40 Ci/mmol^{-1})	0.5	NTB-2	48-72	4		
^{3}H-Glucose (33 Ci/mmol^{-1})	2	NTB-2	12-24	4	Delaware estuary	Elifantz et al. 2005
^{3}H-EPS [from *Nitzschia* grown on ^{3}H-glucose]	1,5	NTB-2	72-144	4		
^{35}S-DMSP (12-43 TBq/mmol^{-1})	<0.1	LM-1	48-144	4	North Atlantic Ocean	Malmstrom et al. 2005
^{35}S-MeSH (43 TBq/mmol^{-1})	<0.01	LM-1	48-144	4		

NAG: N-acetylglucosamine
EPS: extracellular polymeric substances
DMSP: dimethylsulfoniopropionate
MeSH: methanethiol

Product	Description	Company	Art.Nr.:	Size
Agarose	low melting point sea plaque GTGagarose	FMC bioProducts	56111	
Aspartic acid	D-[2,3^3-H]	Amersham Biosience	Nr.: TRK606	250µCi
Aspartic acid	L-[2,3^3-H]	Amersham Biosience	Nr.: TRK574	1mCi
Blocking Reagent	Boehringer Mannheim Blocking reagent	Roche Diagnostics GmbH (NL)	1-096-176	50g
Buechner funnel	(diam.59mm)	VWR International	HALD127C/1	
Citifluor	Glycerol-PBS solution	Citifluor Ltd.	AF1	25ml
DAPI	DAPI stain	Sigma-Aldrich Chemie BV	D9564	1x10mg
Dextran Sulfate		Sigma-Aldrich Chemie BV	D8906	100g
Dispensette	0.5-5ml	Sigma-Aldrich Chemie BV (NIOZ:Brand)	Z33,194-5 (07 X5250?)	
DMF	NN-Dimethyl formamide	Sigma-Aldrich Chemie BV	D4551	250ml
EDTA		Plusone	17-1324-01	
FISHprobes		Thermo Electron Corporation Biopolymers		
Formamide	(Fluka)	Sigma-Aldrich Chemie BV	47671	1l 250ml
Glascribe		Bel-Art Products, Inc. Scienceware	H441500000	
H_2O_2	30% (w/w)	Sigma-Aldrich Chemie BV	H1009	5ml
Kodak Dektol Developer		Sigma-Aldrich Chemie BV	P6917	1GA
Kodak Fixer		Sigma-Aldrich Chemie BV	P6557	1GA
Kodak NBT-2		Integra Biosciences GmbH	IB-01433	1x
Lysozyme	from chicken egg,white	Sigma-Aldrich Chemie BV	Nr.: L7651	50000units/mg 25g
maleic acid		Sigma-Aldrich Chemie BV	M-0375	500g
multiwell plates	12-well (TC treated with lid)	Sigma-Aldrich Chemie BV	M 8687	1St.
Nitril-gloves	Format (blue)	**Unigloves**-Firma Kleiss(in AUT)	Art.Nr.: 6003(M)	100St

Product	Description	Company	Art.Nr.:	Size
Nitrocellulosefilter	0.45µm25mm(diam.)	Sigma-AldrichChemie BV	N9020	100EA
Paraformaldehyde		Sigma-Aldrich Chemie BV	Nr.: P6148	1x500g
Polycarbonate filter	0.2µm 25mm(diam.)	Sigma-Aldrich Chemie BV	P9199	100
Proteinase K	(Fluka) from tritirachium album	Sigma-Aldrich Chemie BV	Nr.: 82456	1x1ml
Safetylight	Dunkel kammer leuchte 230V,50Hz	Kaiser Fototechnik	Nr.: 4018	
Safetylight	lamp model B	Eastman Kodak Company	141-2212	
Safetylight filter	Dunkel kammer filter (rot) 9x12cm	Hama	Nr.:8194	
Safetylight filter	Kodak 2 darkred	Eastman Kodak Company	152-1525	
SDS	Lauryl Sulfate	Sigma-Aldrich Chemie BV	L-4509	100g
2 slide mailer	tubes for emulsion	Raymond A Lamb Limited	Nr.:E6.2	125x
staining dishes		Sigma-Aldrich Chemie BV	S4642	2x3
staining dishes	slide rack	Sigma-Aldrich Chemie BV	S5017	1x3
staining dishes	rack handle	Sigma-Aldrich Chemie BV	S5142	1x3
TEA	Triethyl amine	Sigma-Aldrich Chemie BV	17924	
TRIS		Plusone	17-1321-01	
Triton X100	X-100	Sigma-Aldrich Chemie BV		500ml
Tyramide dye	Alexa Fluor **488** succinimidyl ester	Molecular Probes Europe BV	Nr.: A-20000	1x1mg
Tyramide dye	Alexa Fluor **555** succinimidyl ester	Molecular Probes Europe BV	Nr.: A-20009	1x1mg
TYR-HCl	Tyramine-HCl	Sigma-Aldrich Chemie BV	T2879	5g
Vectashield		Vector Laboratories, Inc.	H-1000	10ml
Water	Sigma Water	Sigma-Aldrich Chemie BV	W4502	1L