CARD-FISH

Introduction: In CARD-FISH, horse-radish peroxidase (HRP) labeled probes are used during the hybridization reaction, which is then followed by an amplification step based on CAtalyzed Reporter Deposition (CARD). This consists of adding fluorescently labeled tyramine molecules to the sample that has been hybridized with HRP labeled probes. The tyramine molecules will activate the horseradish peroxidase on the probes, and thus produce highly reactive intermediates, which are able to bind to moieties of proteins such as tyrosine close to the probe binding site within the cell. In this way, numerous fluorescent molecules will be introduced into the vicinity of the probe target site, which will therefore result in a higher signal intensity and sensitivity.

CARD-FISH can be used to target both multicopy (ribosomal) as well as low copy genes (e.g. housekeeping genes or functional genes), even mRNA. Furthermore, CARD-FISH can be combined with several other microscopical techniques (e.g. standard FISH, polynucleotide FISH, scanning electron microscopy).

You can choose to perform CARD-FISH on your own (see protocols on this and the following pages), or employ kits (e.g. from Molecular Probes, Tyramide Signal Amplification Kits, MP 20911).

Several CARD-FISH protocols can be found on the web, e.g.:

For aquatic samples and sediments:

- Wendeberg, 2010. Fluorescence in situ hybridization for the identification of environmental microbes: http://cshprotocols.cshlp.org/content/2010/1/pdb.prot5366.full?sid=6fdd2780-7c2b-468e-ac10-a660306a5cfa

- www.arb-silva.de/fish-probes/fish-protocols/. Here, various CARD-FISH protocols are listed, for various environmental systems.

For soil:

Schmidt H H, Eickhorst, TT, Tippkötter R. R. 2012. Evaluation of tyramide solutions for an improved detection and enumeration of single microbial cells in soil by CARD-FISH. J Microbiol Methods 91(3):399-405 (2012)

HRP labeled probes can be ordered from e.g. Biomers, http://www.biomers.net/index.html

Detailed, general procedure for CARD-FISH (adapted from T. Reinthaler and E. Teira and Wendeberg, 2010):

Overview of procedure:

Prior to CARD-FISH samples must be fixed (similar concerns with respect to sampling (dilution, concentration, extraction etc) and fixation procedures as with standard FISH) and where appropriate (e.g. membrane filters with concentrated cells from water samples, see e.g. www.arb-silva.de/fish-probes/fish-protocols/ or Wendeberg 2010) embedded e.g. with a protective layer of a 0,1 % agarose solution.

The CARD-FISH procedure consists principally of six steps, spanning over a period of minimum 2 to 4 days, or even longer:

Day 1 and 2:

1) Tyramide synthesis (multiple aliquots can be made once, and then stored for a longer time for subsequent experiments). pp 3-4.

Day 2 (or day 1 if stocks of tyramide are available, self made or from a kit):

2) Permeabilization. pp 4-5.

3) Inactivation of endogenous peroxidases (where appropriate). p.6

4) Hybridization. p. 6-8

Day 3 (or day 2 if stocks of tyramide are available, self made or from a kit):

5) Amplification. p. 8-9.

6) Evaluation by microscope.

Comment: Unfortunately, the CARDFISH protocol applies only for one HRP probe per time. If you wish to employ other HRP probes (multi color CARD-FISH, see Wendeberg, 2010), then you must perform the CARD-FISH experiments subsequently (one experiment per probe). **After each experiment, you must inactivate the HRP from the previous hybridization and then choose another dye for the tyramide for your next probe.**

Day 1-2 (this step can be replaced by a commercial kit):

1. Tyramide synthesis (this is done only once in a while for each type of tyramide dye)

Reagents:

- Tyramide dye, e.g.:
  - **1 mg A488 succinimidyl ester (Molecular Probes Cat. Nr. A-20000) – Fluos/Green**
  - **1 mg A555 succinimidyl ester (Molecular Probes Cat. Nr. A-20009) – Cy3/red**
  - Examples of other options: A350 succinimidyl ester; A546 succinimidyl ester; A633 succinimidyl ester, CF (carboxyfluorescein succinimidyl ester)

  Note: Keep anhydrous, because all succinimidyl esters can hydrolyze rapidly.
- N, N, Dimethylformamide (water free, Sigma D 4551)*
- Tyramine-HCl (Sigma D 4551)
- TEA - Triethylamine (Sigma 17924)*
- Optional: IPBA (p-iodophenylboronic acid, Aldrich 471933)

* Dangerous and sensitive, work in the hood and place on ice.

Day 1:

Solutions to prepare (do not autoclave):

1. **Dye-stock solution:** DMF-TEA (triethylamine): dissolve 1 ml DMF + 10 µl TEA in a 2 ml tube.
2. **TYR-HCl stock solution:** dissolve 10 mg TYR-HCL+1 ml DMF-TEA stock solution in a 15 ml Greyner tube. Prepare just before use.
3. **Preparation of succinimidyl ester:** Mix 1 mg active ester with 100 µl DMF (this is valid for e.g. A488 and A555, check Wendeberg 2010 for other dyes and concentrations). ***Note, esters are light sensitive and thus prone to hydrolysis. Therefore, prepare the solutions shortly before tyramide synthesis, store the esters on ice (with lid) until they can be used for synthesis.***

Synthesis:

a) Depending on label, for example:

- 100 µl $Alexa_{488}$ + 25.2 µl TYR-stock solution,
- 100 µl $Alexa_{555}$ + 12.6 µl TYR-stock solution.

For preparation of other labels, see Wendeberg 2010.

b) Incubate at room temperature in the dark for ~ 6-12 h, slowly rotating.

Day 2:

c) Dilute with $ETOH_{abs.\ (molecular\ grade)}$ to a final concentration of 1 mg (i.e. 1000 µl end volume (1 mg dye/ml). For A_{488}, 874,8 µl, and for A_{555} 887,4 µl).

d) Prepare aliquots (e.g. 20-50 µl) and store at -20°C, or lyophilize and store at room temperature, however, desiccated tyramides can be stored for longer periods (years)

at - 20°C. Protect from light.

e) If you lyophilized your tyramides, reconstitute them in e.g. 20 µL of DMF* (Alexa488, Alexa546, Alexa633, or CF) or in sterile H2O (Alexa350). Store tyramides in DMF at -20°C. Store tyramides in H2O at 4°C. Protect from light.

* You may add 20 mg/ml IPBA to the DMF solution. This may enhance the CARD-FISH signal (Wendeberg 2010).

Day 2 (or day 1 if tyramide stocks are available)

2. Permeabilization (on microscope slides, filters, or in solution)

Solutions to prepare and autoclave:

- 1 M Tris-HCl (156, 6 g/l (Mw 121,14 g/ml), adjust pH to 8 with 6 M HCl.
- 0,5 M EDTA (186,12 g/l (Mw 372,24g/mol), adjust pH with NaOH pastilles (will dissolve only at pH 8).

Prepare ***permeabilization mix*** with either e.g. lysozyme (for e.g. the Bacteria probe) or proteinase K (for e.g. the Archaea probe), store on ice. Where appropriate, consider also other suitable enzymes. For example, achromopeptidase for *Actinobacteria* (see Wendeberg 2010).

Composition of permeabilization mix:

Stock reagent	Volume	Final
Select appropriate enzyme, e.g.:		
- Lysozyme (Sigma, L7651)	100 mg	10 mg/ml
- Proteinase K (Sigma, 82456)	109 mg	10.9 mg/ml
1 M Tris-HCl	1000 µl	0.1 M
0.5 M EDTA	1000 µl	0.05 M
MQ water	8000 µl	……

Procedure:

1) Choose how you wish to handle your sample:

a) Spot and dry fixed cells on a microscope slide. Keep the microscope slide in a tray like a petri dish. Keep the tray moist with a wetted tissue (use your hybridization buffer).

b) Pipette a solution of fixed cells into an eppendorf tube. All replacement of solutions are then done via centrifugation. Cells will not be spotted on a microscope slide until it is time to do microscopy.

If you have membrane filters with cells – whole filters or cut pieces (where appropriate with protective agarose coating!) - you can choose to either place a filter piece on a microscope slide (use tape to position it, and handle the slide as in option a), or place it

into an eppendorf tube and handle it as in option b). Keep track of which side of the filter contains the cells!

2) Add enzyme mix to your sample (ensure that the sample is covered by the enzyme solution). Incubate for 1 h at 37°C.

3) Wash with sterile dest H2O or MQ (1x for lysozyme; 3x for proteinase K) – this is done by moving the slide/filter to a new tray, or by centrifugation of the solution in the tube.

4) Add 0.01 HCl to your sample (ensure that the sample is covered by the enzyme solution). Incubate for 20-25 min at room temperature.

5) Wash with excess dest H2O or MQ.

6) Wash with 96 % EtOH (molecular grade).

7) Dry sample and continue with next step (or store shortly at +4°C or for longer periods at -20°C until further processing).

Day 2 (or day 1 if tyramide stocks are available)

3. Inactivation of peroxidases

This step is only necessary for these two situations:

- Your sample contains a high amount of endogenous peroxidases (e.g. anammox bacteria). You can check for this by performing CARD-FISH *without* HRP probes.

- You are performing multi-color CARD-FISH, employing different types of HRP probes, subsequently (one HRP probe per experimental cycle).

Procedure:

1) Incubate samples with e.g. 0,1 % H2O2 (other concentrations may apply for other samples, see e.g. Pavlekovic et al. 2009. J Microbiol Methods 78(2):8) for 1-2 minutes (or longer) at room temperature.

2) Wash the samples with sterile distilled H2O or MQ for 1 min.

Day 2 (or day 1 if tyramide stocks are available)

4. Hybridization with HRP probes

Start with preparing the hybridization buffer (can be prepared and stored at -20 ° C for up to 1 year), keep on ice during work.

Solutions to prepare and autoclave (exception blocking reagent):

- 5 M NaCl (146,1 g/500 ml (Mw 58,44 g/mol)

- Blocking reagent 10 % (100 ml):

  Step 1:
  - Maleic acid buffer 100 mM (11,608 g/l (Mw 116,08 g/mol).
  - NaCl 150 mM (8,766 g/l (Mw 58,44 g/mol).
  - Adjust pH with solid NaOH to 7.5.

  Step 2:
  - Add Boehringer Mannheim blocking reagent (Roche, Nr 1 096 196) to a final concentration of 10% (10 gram with 100 ml Maleic acid buffer).
  - Dissolve reagent at 60°C with stirring for about 1 h, do not boil.
  - Autoclave.

- Prepare 10 ml aliquots, store them at -20 ° C for longer periods, or at +4 ° C for a few days.

Preparation of hybridization buffer:

Stock reagent	Volume (g/µl)	Final conc.
Dextran Sulfate	1 g	10 %
5 M NaCl	1800 µl	900 mM
1 M Tris-HCl	200 µl	20 mM
100% Triton X100	5 µl	0.05 %

Heat solution at 40-60°C, cool down on ice.

Add Formamide	5500 µl*	55%
or	2000 µl*	20%
10 % Blocking R	1000 µl	1 %
Sigma Water	1500 or 5000 µl	

* Formamide concentration depends on the probe used, e.g. 55 % for e,g. eubacteria, ALF, BET, GAM, CF; and 20 % for e.g. CREN and EUR.

Probes: HRP labeled probes can be ordered from e.g. Biomers, http://www.biomers.net/index.html.

Prepare working solutions of the probe at a concentration of e.g. 50 ng DNA/microliter. Store aliquots (e.g. 50- to 100-µL) at -20°C. Note, HRP probes have limited shelf life. Store aliquots at -20°C. Avoid frequent freezing-thwaing cycles. Store thawed aliquots for up to 6 months at 4°C.

For quality check of probes and measurement of their concentration, see either Wendeberg 2010 or www.arb-silva.de/fish-probes/fish-protocols/.

Consider appropriate controls, e.g. nonsense probe and appropriate reference cells.

Hybridization procedure:

1) Pipette 20 µl of hybridization buffer and 1 µl of HRP labeled probe on a microscope slide well (or, add 300 µl hybridization buffer and 15 µl of HRP labeled probe to a 0,7 ml tube with a filter piece or sample pellet). Probe to buffer ratio should always be 1:20.

2) Hybridise in a moistured chamber/tray/tube at 35°C for 12-15 h, **in dark**. If possible, shake/rotate slowly (e.g. stick tubes around a rotor).

3) Prepare washing buffer, as follows:

Stock reagent	Volume (µl)	Final
5 M NaCl	30*	13 mM
	1350**	
1 M Tris-HCl	1000	20 mM
0.5 M EDTA	500	5 mM
MQ water	48420*	
	47100**	
10 % SDS	50	0.01 %

* For 55 % Formamide; ** For 20 % Formamide. If other formamide concentrations are needed, calculate the corresponding proportions.

Solution to prepare: 10 % SDS (dissolve 50 g in 500 ml MQ, dissolve at 40 ° C in water bath, do not autoclave.

4) Prewarm washing buffer at 37°C.

5) Transfer microscope slide/sample solution/filters quickly to washing buffer (or pipette hybridization solution to the tube with the pellet of sample. Wash for 15 min at 37°C. Dry (e.g. either with pressurized air or on a buechner funnel).

Day 3 (or day 2 if tyramide stocks are available)

5. Amplification (on microscope slides, on filters or in a tube)

Start with preparing the PBS-T mix.

Solutions to prepare and autoclave:

1x PBS:	A) Na_2HPO_4	200 mM
	B) NaH_2PO_4	200 mM
	Adjust pH to 7.2-7.4 of solution A with solution B	
	1x PBS: NaCL	130 mM
	Na_xPO_4	10 mM
	A. dest ad 1000 ml	

Comment: The pH of 1x PBS should be 7,4-7,6 (for better peroxidase turnover)

PBS-T:	1X PBS	49750 µl
	100 % Triton X100	0.05 %

Stock reagent	Volume (µl)	Final
1xPBS	49750	
100% Triton X100	25	0,05 %

Amplification procedure:

1) Incubate microscope slide/filter/tube with sample in PBS-T mix at room temperature for 10-15 min.

2) Meanwhile prepare amplification buffer (AMP, store at +4 ° C).

Stock reagent	Volume	Final
Dextran Sulfate	2 g	10 %
5 M NaCl	8000 µl	2 M
10 % Blocking R	200 µl	0.1 %
1X PBS	11800 µl	…..

Heat in water bath to dissolve.

3) Prepare substrate mix with prepared amplification buffer (AMP) and 30 % H_2O_2. Dilute 30 % H_2O_2 to final concentration of 0,0015 % (Step A to B; all volumes in µl):

Tube	AMP	H_2O_2	Tyr	Ratio	Dye
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

For other dyes, see procedure in e.g. Wendeberg 2010.

Short guidance:

a. prepare two tubes: A and B.
b. Pipette into tube A: 200 µl of amplification buffer and 1 µl of 30% H_2O_2.
c. Pipette into tube B: 493 µl amplification buffer, 5 µl from tube A and 2.5 µl of $Alexa_{555}$, or 3.4 µl of $Alexa_{488}$.

4) Pipette 15 µl substrate mix onto slide and incubate at 37°C for 10 – 45 min, dark.

5) Wash slide/filter in 50 ml PBS-T mix at room temperature for 10 min in the dark.

6) Wash with dH2O or MQ; then wash with 96 % $ETOH_{abs.\ (molecular\ grade).}$

7) Dry and mount in a mounting-medium such as Citifluor (with or without a nucleic acid stain such as DAPI or SYBR Green, see example below):

Stock reagent	Volume (µl)	Final
DAPI 50 µg/ml	40	1 µg/ml
1x PBS	140	0,5
Citifluor*	1820	

*Or use a mix of different mounting media, e.g.Vectashield 280 µl and Citifluor 1540 µl.

Comment: Samples can also be stored at dark in the fridge for a period prior to microscopy.