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HCR-FISH for Choanoflagellate Cultures

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1 Works for me This protocol is published without a DOI.

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ABSTRACT

This protocol is a fluorescent *in situ* hybridization (FISH) technique for labeling 16S rRNA from specific bacteria in choanoflagellate cultures. It combines the fixation, cell mounting, and washing methods used in traditional FISH and catalyzed reporter deposition (CARD) FISH (Glöckner et al., 1999; Pernthaler et al., 2002), with the more permeablie *in situ* hybridization signal amplifcation technique of hybridization chain reaction (HCR) FISH (Choi et al., 2016; DePas et al., 2016). This is the first successful FISH method in choanoflagellates, and it provides a good foundation for future adaption for mRNA and other FISH methods in choanoflagellates.

PROTOCOL CITATION

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Choanoflagellate, bacteria, CARD-FISH, HCR-FISH

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BEFORE STARTING

Make all necessary buffers ahead of time (except Wash Buffer which you make fresh each time you need it).

Hybridization Buffer:

Reagent	Per 1 ml	Per 50 ml
20X SSC (Nuclease free)	100 µl	5 ml
Dextran Sulfate	100 mg	5 g
Formamide (final conc. 20%)	200 µl	10 ml
Nuclease free H2O - Fill to:	1 ml	50 ml

1. Heat to 45˚C to help the dextran sulfate solubilize.
2. Filter through a 0.2µm filter once buffer is in solution.
3. Store at -20˚C when not in use.

- NOTE: This hybridization buffer is modeled after Depas et al. (2016) and it is designed for short probes targeting 16S rRNA. Targeting mRNA with longer prober requires a different strategy and hybridization buffer.

Learn more at Molecular Instruments.

- NOTE: Most short probes targeting 16S rRNA work best with a formamide concentration of either 20 or 25%. It helps to try both when testing new probes.
- NOTE: Dextran Sulfate - Large (>500,00 MW) Sigma D6001

Wash Buffer: (Produce freshly right before use, final volume 50 ml)

1. 0.5 ml 0.5M EDTA, pH 8.0
2. 1.0 ml 1M Tris HCl, pH 8.0
3. X µl 5M NaCl (Depends on formamide concentration, see table)
4. Add nuclease free H_2O to a final volume of 50ml.
5. Add 25 µl SDS (20% w/v). Add SDS last to prevent precipiation.

% Formamide	µl of 5M NaCl	% Formamide	µl of 5M NaCl
0	8900	35	700
5	6260	40	460
10	4400	45	300
15	3080	50	180
20	**2150**	55	100
25	**1490**	60	40
30	1020		

The stringency in the washing buffer is achieved by adjusting the NaCl concentration based on the formamide concentration used in the hybridization buffer. These concentrations were calculated using the formula from Lathe et al., 1985.

Amplification Buffer: (Make a large batch and store at 4˚C)

1. Make 50% Dextran sulfate by dissolving 25g of Dextran sulfate in 50ml of nuclease free H_2O. Heat to 65˚C to help get it into solution (or let sit overnight at RT).
2. Filter 50% Dextran sulfate solution through a 0.2µm filter and store at -80˚C unless you are using it right away. Leave setup to filter overnight because it is a slow process.
3. Make 250ml of Amplification buffer based on the recipe in the table below.
4. Sterile filter with a 0.2µm media bottle and store at 4˚C.
5. Always use aliquots and keep the big bottle sterile.

Amplification Buffer	for 40 ml	for 250 ml
20X SSC (Nuclease free)	10 ml	62.5 ml
50% Dextran Sulfate	8 ml	50 ml
10% Tween20	400 µl	2500 µl
Fill with nuclease free H2O to:	40 ml	250 ml

Amplification buffer from the HCR-FISH protocol. Choi et al. (2016) Development

Overview of HCR-FISH targeting 16S rRNA.

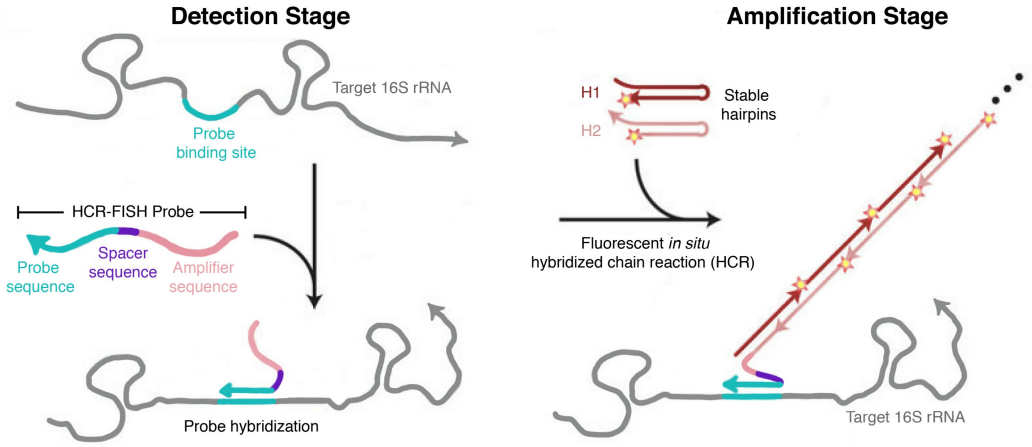

Image modified by Kayley Hake from Choi et al. (2010) Nature Biotechnology.

FIX Choanoflagellate culture

1

1. Fix choanoflagellate culture containing choanoflagellate cells and free-living bacteria for 12-18 hours in fresh 2% paraformaldehyde at 4˚C. (No need to wash or concentrate the culture)

FILTER Choanoflagellate culture

2

1. Using a 15ml glass microanalysis filter holder (Millipore Sigma), filter 250-1000µl of fixed culture onto a filter. To capture all choanoflagellate and free-living bacteria, use a 0.2µm pore size 25mm filter (GTTP02500, Millipore Sigma). To capture only choanoflagellate colonies, use a 5µm pore size 25mm filter (TMTP02500, Millipore Sigma). To help preserve morphology, apply the least amount of vacuum as possible, or let gravity filter the cultures.
2. To assess density of the culture on the filter, carefully look at a filter under a cell culture microscope. Large choanoflagellate colonies are easy to visualize with 10-20x objectives, but smaller colonies are not. If it is hard to make out the choanoflagellates, stain the filters for 5 minutes in 0.1mg/mL Hoechst 33342 (Thermo Fischer) in sea water.
3. Based on the density of the first filter you look at, adjust the amount of culture being added or keep the amount the same and finish filtering your fixed culture.
4. Let filters air-dry face up (cells up) on clean whatman filter paper and label the rim with a pencil to mark the culture information that was filtered. Keep your labeling small, because you will need to add additional labels in the future.
5. Filters can be stored at -20˚C until needed, or continued onto the next step. Filters are stable for many months at -20˚C and should be stored inside a clean piece of folded whatman filter paper in an air tight container.

COAT Filters in agarose

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1. Prepare 0.1% low melt agarose with nuclease free H_2O and sterile filter through a 0.2µm filter. Allow the agarose to cool to 35-40˚C before using.
2. Tape down a clean sheet of parafilm onto a desk.
3. Pipette 2 ml of 0.1% low melt agarose onto the parafilm creating a puddle the size of a 25mm filter.
4. Using tweezers to hold only the rim of the filter, dip the backside of the filter (side without cells) into the 0.1% low melt agarose first followed by the topside (side containing cells).
5. Place the coated filter FACE DOWN (side containing cells) onto a clean portion of the taped down parafilm.
6. Use separate puddles of 0.1% low melt agarose for different cultures to limit contamination between samples.
7. Let filters dry at room temperature (1-2 hours). It is easy to tell when the 0.2µm filters are dry because the color will change from grey back to white.
8. Soak the filters in 2ml of 96% ethanol for 1 min to float filters off of the parafilm.
9. Carefully peel off the filters using tweezers to grab only the rim of the filter, and place the filter FACE UP on a clean piece of whatman filter paper to air-dry.
10. Coated filters can be stored at 4˚C for a few months. Store filters inside a clean piece of folded whatman filter paper in an air tight container.

HCR-FISH: PERMEABILIZATION

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1. Before you cut the filters for each sample or probe combination, label the rim of the 25mm filter in pencil on the side containing the cells with the sample number and approximate placement for each wedge. Write only the number, and provide more detail corresponding to those numbers in your lab notebook. Make sure to label in away that makes the filter orientation clear (ie. instead of 8 write 08).
2. Using tweezers in one hand to hold the filter, carefully cut filters into the desired wedges using disecting scissors (typically 8-10 wedges per 25mm filter). Cut filters over a clean piece of whatman paper to collect wedges as they fall.
3. Use tweezers to grab only the labeled rim of each wedge, place up to 10 wedges in a 2 ml tube with the point facing down (the flat bottom is important for proper distribution of filters in buffers).
4. Prepare fresh CARD-FISH proteinase buffer in a separate tube (make 2 ml of Proteinase Buffer per 2 ml tube) and transfer into the tube containing the filters once it is made. Make sure all of the filters are submerged in the CARD-FISH Proteinase Buffer, and flick the bottom of the tube gently to remove any air bubbles on the filters.
5. Incubate for 30 min. at 37˚C.
6. Wash 2x for 1 min in nuclease free H_2O at room temperature. (Be careful when pipetting to not touch the filters or accidently get them caught on the filter tip).
7. Wash 1x in 98% EtOH and let filters air-dry on clean Whatman filter paper at room temperature.

CARD-FISH Proteinse Buffer

Reagent	Per 2 ml
0.5M EDTA	0.2 ml
1.0M Tris-Cl pH 8.0	0.2 ml
Nuclease free H2O	1.6 ml
Lysozyme	20 mg

HCR-FISH: PROBE HYBRIDIZATION

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1. Transfer filters to a clean 2ml tube and add enough hybridiation buffer to completely cover the filters.
2. Pre-hybridize filters in hybridization buffer for 30 min. at 45˚C. (Can keep filters grouped at this point)(Hybridization buffer recipe can be found in the "Before Start" section).
3. Add 0.25µl of 100mM stock of HCR-FISH probes to 0.5ml of hybridization buffer to a new 2ml tube and vortex to mix. (Each probe combintion should be in it's own tube)(Information on how to design custom HCR-FISH probes can be found in our protocols.io called "16S rRNA probe design for HCR-FISH")
4. Pre-warm hybridization buffer and probe solution to 45˚C.
5. Add filters to their respective probe solutions.
6. Incubate overnight at 45˚C.

HCR-FISH: PROBE WASH

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1. Make fresh probe wash buffer with appropriate NaCl concentration based on the formamide% (See recipe below and the "Before Start" section for more information)(Add SDS last to prevent precipitation).
2. Pre-warm fresh wash buffer to 48˚C.
3. Replace hybridization and probe solution with 2ml of pre-warmed wash buffer to remove excess and unbound probe.
4. Incubate for 1 hour at **48˚C**.
5. Remove probe wash buffer and wash filters 3 x 5 min. with 5X SSCT at room temperature.

Wash Buffer (for 20% Formamide Concentration)

Reagent	Per 50 ml
0.5M EDTA, pH 8.0	0.5 ml
1.0M Tris HCl, pH 8.0	1.0 ml
5M NaCl*	2150 µl
Fill with nuclease free H2O to:	50 ml
20% SDS (w/v)**	25 µl

* The amount of NaCl is dependent on the concentration of formamide in the hybridization buffer. The given amount is for a 20% formamide hybridization buffer. See the "Before Start" for more information.

** Add the SDS last to prevent precipitation.

5x SSCT *(For 40 ml)*

Reagent	Per 40 ml
20X SSC (nuclease free)	10 ml
10% Tween 20	400 µl
Fill with nuclease free H2O to:	40 ml

HCR-FISH: FLUORESCENT HAIRPIN PREPARATION

7 1. Filtersin 500µl of amplification buffer for 30 min. at room tempearture. (Amplification buffer recipe can be found in the "Before Start" sectionHairpin SolutionH1B)
2. While the filters are in the amplification buffer, calculate how much fluorescent HCR hairpins you will need and prepare them for the next steps. HCR hairpins have the fluorophore attached to them and require two hairpins per color to self-assemble during the signal amplifcation step (See the diagram in the "Before Start"). HCR hairpins are pre-diluted in hairpin solution that can be purchased through Molecular Instruments or synthesized by hand following their protocols.
3. In the King lab we have six different HCR hairpins that assemble in pairs into 3 different colors (Table below). For each hairpin color, their is an amplifier sequence that we've assigned. (For more information on amplifier sequences, see our protocol.io on how to design custom HCR-FISH probes called "16S rRNA probe design for HCR-FISH")

HCR Hairpin Colors and Names

Hairpin Fluorophore	Amplifier Sequence	Hairpin 1 Solution	Hairpin 2 Solution
488	B1	B1H1	B1H2
594	B2	B2H1	B2H2
647	B3	B3H1	B3H2

Note: The combination of amplifier sequence with fluorophores is what we chose for the King Lab. You can select your own combinations from Molecular Instruments.

1. In the signal amplification step we are preparing for, the hairpins will be diluted in amplification buffer. To calculate the amount of hairpins you need, you first need to calculate the amount of hairpin cocktail you will be making (amplification buffer + hairpin solution).
2. To better explain, I will use an example experiement with 6 filters that have been hybridized with 3 probes requireing 3 colors (488, 594, and 647).
3. For each filter, you will use 25µl of cocktail. So for 6 filters you will need 150µl of cocktaill. To limit the chance of running out in the end, add an additional 10µl to the cocktail making the final volume 160µl.
4. You will use 1µl of hairpin solution for every 50µl of cocktial solution. This means for a cocktail with 160µl(160/50= 3.2) you need 3.2µl of each hairpin solution, and for cocktail 2, you need 4.2µl.
5. Although you need to keep each hairpin solution separate, you can group the same hairpin solutions you need for both cocktails together resulting in the following total amount of hairpin solutions to be used in this experiement.

Total amount of hairpin solutions needed:

Fluorophore	Hairpin Solution	Amount needed
488	B1H1	3.2µl
488	B1H2	3.2µl
594	B2H1	3.2µl
594	B2H2	3.2µl
647	B3H1	3.2µl
647	B3H2	3.2µl

1. Once you calculate the amount of hairpins you need, pipette the amount of hairpin solution into individual tubes.
2. To prepare the hairpins by snap cooling, heat the hairpins to 95˚C for 90 seconds and let them cool in the dark at room temperature for 30 min.
3. While the hairpins are cooling, calculate the amount of amplification buffer you need by subtracting the volume of total hairpins solutions from the cocktail volume.

Calculations for the amount of Amplification Buffer needed

Cocktail Volume	Minus (# of hairpins)x(amount of hairpin solution)	Amount of Amplification Buffer
160 µl	(6 hairpins)(3.2µl of hairpin solution)= 19.2µl	140.8 µl

HCR-FISH: HAIRPIN HYBRIDIZATION

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1. After the hairpin solutions have cooled for 30 min., make the hairpin cocktail by adding all the respective hairpin solutions to the amount of amplification buffer. (ie. Add 3.2µl for each of the 6 hairpin solutions to 140.8 µl of amplification buffer.)
2. In an air tight container, tape down a fresh piece of parafilm at the bottom.
3. Drop out 25 µl of hairpin cocktail (amplification buffer + hairpin solution) onto the parafilm for each filter. Give enough space between the drops to fit the filters.
4. Using tweezers to grab the labeled rim, carefully place each filter face down (cells down) on their respective drops.
5. Add a wet kimwipe to the corner or side away from the filters in the chamber to keep it humid.
6. Seal the container with parafilm and place it in the dark at room temperature.
7. Incubate overnight.

HCR-FISH: HAIRPIN WASH

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1. Using tweezers, carefully combine 6-10 filters into a 2 ml tube with 2 ml of Amplification Buffer.
2. Incubate for 1 hour in the dark at room temperature to wash away unbound hairpins.
3. Wash 2x 30 min. in 5x SSCT in the dark at room temperature.
4. Wash 1x 10 min. in 5X SSCT + Hoechst 33342 diluted 1:1000 at room temperature in the dark.
5. Wash 1 minute at room temperature in nuclease free H_2O.
6. Wash 1 minute in 96% EtOH.
7. Let the filters dry on whatman paper.

HCR-FISH: SAMPLE MOUNTING

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1. Using a pipette tip that has been cut at the end for viscous solutions, pipette a large smear of ProLong Diamond (Molecular Probes) onto a coverslip. For 24x40-1 coverslips (12-545-D Fisher), I place ~125µl of mounting solution.
2. Using tweezers, carefully place filters **cells down** onto the smear of Prolong Diamond. (Mount 4-6 filters on a 24x40 coverslip)
3. Place a similar smear of mounting solution onto a glass slide.
4. Carefully flip the slide onto the coverslip by lowering the slide slowly from above until the mounting solutions from each side touch. Lower slowly to let the mounting solution spread out and prevent air bubbles.
5. Leave slides to set overnight in the dark at room temperature.