Hybridization Chain Reaction 3.0 In *Platynereis*

Adapted from protocols by D Arendt, BD Özpolat, F Raible, G Wessel, and HMT Choi  
V.0.3\_2020.Nov

Bria’s comments and changes written in this color

# Overview:

# This protocol outlines how to design and carry out multiplexed, whole mount mRNA *in situ* hybridization using the HCR V3.0 methodology in fixed annelid specimen.

## Reagents needed

### CHEMICALS

**Reagent CAS# Vendor Vendor Number Location**

* MilliQ Water
* Tris-HCl R-Shelf
* Glycerol R-Shelf
* Tween-20 R-Shelf
* Proteinase K R-Frzr1
* DAPI R-Frzr1
* DEPC
* NaCl
* Trisodium Citrate
* Glycine

### SOLUTIONS/ALIQUOTS

* Proteinase K (10mg/mL) Rowe Lab Freezer #1
* DAPI (5mg/mL) Rowe Lab Freezer #1
* IDT oPools (1pmol/uL in Tris pH7.5) Rowe Lab Freezer #2
* Amplifier Hairpins (3pmol/uL in 5XSSC) Rowe Lab Freezer #1
* Hybridization Buffer, Probe Wash Buffer Rowe Lab Freezer #1
* SlowFade Glass (Thermo [S36917-5X2ML](https://www.thermofisher.com/order/catalog/product/S36917-5X2ML)) Rowe Lab Freezer #2
* Amplification Buffer Rowe Lab 4C
* 1X DEPC-PBST Room Temp
* 5X SSC
* 5X SSCT Room Temp
* 25% Glycerol, 50% Glycerol, 75% Glycerol in DEPC-PBS Room Temp

### CONSUMABLES

* PCR tubes, 1.5mL Eppendorf tubes, 2.0mL Eppendorf tubes
* Dark box/drawer
* 6 well plate
* Transfer pipettes or pasteur pipettes

## Solutions and Stocks

### Saline Sodium Citrate Buffer (SSC) - 20X

* 175.3g [NaCl](http://en.wikipedia.org/wiki/Nacl)
* 88.2g [trisodium citrate](http://en.wikipedia.org/wiki/Trisodium_citrate)
* 800ml dH2O (RNase free if required)

*adjust the pH to 7.0 with a few drops of 1M HCl  
adjust the volume to 1L with dH2O  
sterilize by autoclaving*

### 5X SSC/SSCT Concentrate

* 12.5**mL** 20X SSC Buffer
* 5.00**mL** 10% Tween-20 (Add if making SSCT, if making SSC replace with 5mL H2O)
* 32.5**mL** MilliQ H2O

### 10X PBS Concentrate

* 2.56**g** NaH2PO4 Monohydrate (Sodium phosphate monobasic)
* 14.97**g** Na2HPO4 Anhydrous (Sodium phosphate dibasic anhydrous)
* 102.2**g** NaCl (Sodium chloride)

*Mix all powders in 800 mL dH20, adjust pH to 7.4 with HCl or NaOH.*

*Add remaining water (to make 1L total).*

***Autoclave.***

### Proteinase K Stock Solution – 10mg/mL, 1%w/w, 10X

* 20.0**mg** Proteinase K
* 2.00**mL** MilliQ water

*Use 15mL Falcon tube (but don’t use cap as a weigh boat)*

*Aliquot 200uL per Eppendorf with 5mL Combitip*

*Store at -20C*

### DEPC Treated Water

* 1**mL** DEPC
* 1**L** MilliQ water

*DEPC is carcinogenic before autoclaving, so pipette under hood.*

*Mix bottles thoroughly.*

***Autoclave.***

*Solutions made with DEPC-treated H2O don’t need to be autoclaved.*

## Protocol

### Probe Design (allow 1 week for manufacture and arrival)

*Probes can be ordered from Molecular Instruments. However, to have more control over what sequences are used we have written a python script that allows us to design and order probes that work with the HCR 3.0 system. The latest release can be found at* [*https://github.com/rwnull/insitu\_probe\_generator/releases*](https://github.com/rwnull/insitu_probe_generator/releases)

1. Generally speaking it seems that as few as 5 probe pairs up to 40 pairs work, with the ideal number sitting between 20 and 30 pairs
2. That said, the length of transcript being visualized will dictate how many probes can be made. Each probe pair covers 54 base pairs, so a transcript of 1000bp cannot possibly fit 40 probe pairs.
3. We hypothesize that probes that are low complexity (e.g. large homopolymeric runs) or terminal GC-rich regions may lead to spurious hybridization and increase the likelihood of background amplification. So, reviewing sequence output is a good practice.
4. Once happy with the probe sequences, they can be submitted to IDT for manufacture.
   * 10pmol is generally sufficient for 5 reactions at 2pmol/reaction.
5. Resuspend at 1pmol/uL (1uM) in a neutral pH, nuclease-free water or Tris-buffered DNA storage solution, store at -20C
6. IDT-ordered hairpins can be resuspended at 3pmol/uL (or at 30pmol/uL as 10X stock) in 5xSSC and stored at -20C

### Tissue Collection (>48hrs before DAY 1)

*To make tissue harvesting easier utilize Anesthetizing Seawater (Mg+FSW)*

1. Set up a dissection dish with **MgCl2-Containing seawater**
2. Select the animals to be sampled and place them in the **Mg+FSW**
3. Allow sufficient time for the animals to become immobilized **~15 min**
4. Make amputation/manipulation
5. Fix according to regular protocol, step through MeOH series if long term storage

### HCR DAY 1 - Rehydration, ProK, PostFix, Probe Hybridization

### *Adapted from “Whole-mount* [*in situ hybridization protocol*](https://docs.google.com/document/d/1qzcEUxV_0DzeoYpnIqLq2nvN2eGjCST6/edit) *for Platynereis samples for 3D microscopy” Group Balavoine/Vervoort (16/1/14), Institut Jacques Monod, Paris, adapted from Jékely Lab and HCR v3.0 protocol for whole-mount* [*nematode samples*](https://files.molecularinstruments.com/MI-Protocol-HCRv3-Nematode-Rev6.pdf)

**\\NOTE\\** It is possible to combine specimens from multiple stages AFTER permeabilization and post-fixation. This allows you to conserve reagents and time.

Volumes by various containers:

* Larvae in baskets in 2mL tubes: 500uL
* Juveniles in 2mL tubes: 700uL
* Juveniles in 6 well plates: 4mL
* Tails in 12 well plates: 1.5mL

Where N is the number of groups (tubes, wells, etc).

* **Rehydrate tissue with 5min washes through decreasing MeOH gradient (75%,50%,25%,PT,PT)**

Keep tissue on ice and encourage rehydration by utilizing an orbital shaker at ~120rpm

2mL Eppendorfs work well for larvae in baskets and juveniles once separated into groups. 6 well plates are useful for older stages. Volumes for various containers are calculated [here](https://docs.google.com/spreadsheets/d/19bRDT_oK3G96_LU7VF-a0Dm3Pesg8yTqSmvPp-Ql6GI/edit#gid=0).

* **Prepare 3 solutions in the following volumes per sample *fresh before each experiment*:**

(1) Proteinase K: 10 μl of 20mg/ml ProK in N volumes PTw, for a final concentration of 0.1mg/mL. -- Keep at Room Temp.

(2) Glycine 2mg/ml in N volumes PTw -- Keep on Ice.

(3) 4% paraformaldehyde in N volumes PTw -- Keep on Ice.

* **Digest samples with Proteinase-K without shaking at RT:**

|  |  |  |
| --- | --- | --- |
| **Animal/Stage** | **Proteinase K Concentration** | **Digestion Time** |
| Platynereis -- <1 day pf | 0.1 mg/ml final concentration | 30 seconds |
| Platynereis -- 1-2.5 days pf | 0.1 mg/ml final concentration | 60 seconds |
| Platynereis -- 3-5 days pf | 0.1 mg/ml final concentration | 2 minutes |
| Platynereis -- 1-6 weeks pf | 0.1 mg/ml final concentration | 3 minutes |

* + From Gazave et al 2013 : Extensive proteinase K incubations were performed for later stages (3 min for 5 days and 4 min for 15 days, 0.1 mg/ml).
  + Emily Kuehn’s juvenile experiments used a 4min incubation
  + Bria Metzger’s experiments with 10 segment -> mature worms used 4min incubations
  + Juveniles with blastema are treated according to age of *blastema* as above
* **1X PTw quick wash to remove bulk of ProK** I’ve been doing this as a ‘rinse’ — solution in, solution out.
* **Wash samples with 1 volume 2mg/ml glycine in PTw 15min. -- Keep on Ice.**
* **Wash 2x in 1 volume PTw**
* **Post-fix samples in 4% PFA in PTw on ice for 30 minutes, orbital shaking at ~120rpm. Transfer to 2mL tubes after this step.**
* **Wash 5x 5min in 1 volume PTw, shaking at ~120rpm. On Ice.**
* ***Detection stage***

**\\NOTE\\** This is a good place to combine samples of differentiable stages into a single tube.

1. Incubate samples in **1 volume** of 50% PBST / 50% probe hybridization buffer for >5 min at room temperature.

**//CAUTION//** Probe hybridization buffer contains formamide, a hazardous material.

**\\NOTE\\** Younger stages may float, and require longer than 5 min to equilibrate.

**||Optional||** Centrifuge at 200 × g for 2 min to sediment tissue.

2. Remove the solution.

3. Pre-hybridize samples in 300 µL of probe hybridization buffer at 37 ◦C for 1 h.

4. Prepare probe solution by adding **4 pmol** of each probe mixture (e.g. 2 µL of 1 µM stock) to 200 µL of probe hybridization buffer at 37 ◦C.

**\\NOTE\\** For digital HCR imaging, use higher concentration of probe to improve probe hybridization efficiency.

5. Add the probe solution to reach a final hybridization volume of 500 µL.

6. Incubate samples overnight (12–16 h) at 37 ◦C.

### HCR DAY 2 - Washes, Preamplification, Signal Amplification (optional: DAPI)

**\\NOTE\\** equilibrate amplification buffer to room temperature before use.

**\\NOTE\\** pre-heat probe wash buffer to 37 ◦C before use.

***Detection stage, continued***

7. Remove excess probes by washing samples 4 × 15 min with 1 mL of probe wash buffer at 37 ◦C.

**//CAUTION//** probe wash buffer contains formamide, a hazardous material.

**||Optional||** Bring samples to the bottom of the tube by centrifuging at 500 × g for 2 min for each wash.

**||Optional||** **Save probes for future use.**

8. Wash samples 2 × 5 min with **1 volume** of 5× SSCT at room temperature.

***Amplification stage***

1. Pre-amplify samples with 300 µL of amplification buffer for 30 min at room temperature.

2. Separately prepare 30 pmol of hairpin h1 and 30 pmol of hairpin h2 by snap cooling 10 µL of 3 µM stock in Thermocycler (heat at 95 ◦C for 90 seconds and cool to room temperature in a dark drawer for 30 min).

**\\NOTE\\** HCR hairpins h1 and h2 are provided ready for snap cooling, and should be snap cooled in separate tubes. Do not combine H1s and H2s from various B1s, B2s etc either.

3. Prepare hairpin mixture by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to 200 µL of amplification buffer at room temperature.

**||Optional||** Add DAPI to a final concentration of **2ug**/mL to stain nuclei.

4. Add the hairpin mixture to reach a final amplification volume of 500 µL.

5. Incubate the samples 2days (48 h) in a blacked-out water bath at 26◦C.

**\\NOTE\\** For digital HCR imaging, amplify for a shorter period of time to ensure single-molecule dots are diffraction limited.

### HCR DAY 3 - Amplification termination, SlowFade -or- Glycerol series (optional: DAPI)

**\\NOTE\\** equilibrate SlowFade Glass to room temperature for 1hr prior to use.

1. Remove excess hairpins by washing with 1 mL of 5× SSCT at room temperature:

(a) 2 × 5 min

(b) 2 × 30 min

(c) 1 × 5 min

**||Optional||** bring to the bottom of the tube with centrifugation at 500 × g for 2 min for each wash.

2. Mount sample on slide with SlowFade Glass according to [manufacturer guidelines](https://drive.google.com/file/d/1YoTxuE8ZWpuFPffYHAQdrfLUoSxCXoru/view?usp=sharing).

* For juveniles and mature worms, mount tails separately to image the PGZ effectively. Cut off the final few segments of each worm and mount in the same order.
* Mount between two coverslips, one long and one short. Refractive index matching is not so important here, so the smaller square coverslips are fine for smaller samples.
* Clean the coverslips with a kimwipe.
* Gently scrape each corner of the small coverslip through mounting clay until each corner has a decent sized bead. Set aside, clay-side up.
* Transfer to the slide:
  + For juveniles: tip contents into a small dish. Using a pair of tweezers, move the worm from the dish to the slide by gently grasping the head (or another region, if you want to preserve head morphology).
  + For larvae: tip out contents onto the slide. Looking under the dissecting microscope, use a p10 to pipette away as much excess water as possible.
* Add slowfade glass:
  + For juveniles: Bring the droplet into contact with the glass. Then, keeping gentle pressure to keep the slowfade flowing, outline each worm. If needed, add a little more between multiple worms mounted on the same slide. Use a p10 (barely any pressure) to remove any bubbles.
  + For larvae: Bring the droplet into contact with the glass. Then, keeping gentle pressure to keep the slowfade flowing, draw the tip in a circle bounding the larvae on the slide. If needed, fill in the center. Use a p10 (barely any pressure) to remove any bubbles.
* Place the coverslip. Gently press one edge of the coverslip down, then the opposite edge.
* Place the slide and coverslip on the microscope stage. Use the corner of a thick glass slide (with purple label) to apply gentle pressure to each corner of the coverslip. Rather than pressing straight down, it helps to apply pressure in a small stroke towards the outer edge.
* Label the slide with the orientation of the worm (ventral/dorsal), keeping in mind that the confocal is inverted.
* Seal around the edges with nail polish.
* Label with the experiment number (ex. HCR 5), group ID, worm number (ex. worms 3, 4), and date.
* Place in a slide folder and keep at 4C until ready to image.

References

*Choi et al. 2018.* PMID: 29945988 [Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust](https://dev.biologists.org/content/145/12/dev165753.long)

*Gazave et al 2013.* PMID:23891818 [Posterior elongation in the annelid *Platynereis dumerilii* involves stem cells molecularly related to primordial germ cells](https://www.sciencedirect.com/science/article/pii/S0012160613003813?via%3Dihub)

*Invitrogen/ThermoFisher 2020.* PubNo MAN0018916 Rev: B.0 [SlowFadeTM Antifade Mountants](https://drive.google.com/file/d/1YoTxuE8ZWpuFPffYHAQdrfLUoSxCXoru/view)

*Molecular Instruments 2020.* Rev: 6 [MI-Protocol-HCRv3-Nematode](https://files.molecularinstruments.com/MI-Protocol-HCRv3-Nematode-Rev6.pdf)

*Group Balavoine/Vervoort 2014.* Rev: Jan16 [Whole-mount in situ hybridization protocol for Platynereis samples for 3D microscopy](https://docs.google.com/document/d/1qzcEUxV_0DzeoYpnIqLq2nvN2eGjCST6/view)