**HYBRIDIZATION CHAIN REACTION IN *PRISTINA LEIDYI* V.1**

Based on Ryan´s *Pristina* HCR experiment and Bria´s HCR in *Platynereis* protocol:

* **From fixation to post-fixation:** Alexa Bely *Pristina in situ* *Protocol* 11/08/21
* **Hybridization & amplification:** Molecular Instruments *C.elegans* HCR protocol & Bria HCR in *Platynereis* protocol.

**REAGENTS**

**Reagent Location**

* Tris-HCl 309/Room Temp Shelf
* Tween-20 309/Room Temp Shelf
* **Pronase E (10 mg/mL)** 309/Freezer Shelf #3
* **DAPI (5 ug/uL)** 309/Freezer Shelf #3
* DEPC **(TOXIC: Carcinogenic)** 309/Room Temp Shelf
* NaCl 309/Room Temp Shelf
* Trisodium Citrate 309/Room Temp Shelf
* Glycine (powder) 309/Room Temp Shelf
* 0.2M Triethanolamine **(TOXIC: N-Azide)** 313/Black Freezer
* Acetic anhydride **(TOXIC: Highly corrosive)**  313/Yellow cabinet
* IDT oPools (1 pmol/µL in DEPC-water) 309/Freezer Shelf #2
* Amplifier Hairpins (3 pmol/µL in 5xSSC) 309/Freezer Shelf #3
* Probes Hybridization Buffer **(TOXIC: 10-30% Formamide)** 309/Freezer Shelf #3
* Probe Wash Buffer **(TOXIC: 10-30% Formamide)** 309/Freezer Shelf #3
* Amplification Buffer **(TOXIC: 10-30% Formamide)** 309/4ºC Refrigerator
* SlowFade Glass with DAPI (Thermo S36920-5X2ML) 309/Freezer Shelf #3
* 1x DEPC-PBSt 309/Room Temp Shelf
* 5x SSCt 309/Room Temp Shelf

**CONSUMABLES**

* 2.0 mL Eppendorf tubes
* 15 mL Falcon tubes
* Dark box/drawer
* 6 well plates
* Transfer pipettes or pasteur pipettes

**SOLUTIONS AND STOCKS**

**Saline Sodium Citrate Buffer (SSC) 20x:**

* 175.3 g [NaCl](http://en.wikipedia.org/wiki/Nacl)
* 88.2 g [trisodium citrate](http://en.wikipedia.org/wiki/Trisodium_citrate)
* 800 mL 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:**

* 12.5 mL 20X SSC Buffer
* 2.5 mL 10% Tween-20 (Add if making SSCT, if making SSC replace with 2.5mL H2O)
* 35 mL MilliQ H2O

**10x PBS:**

* 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.***

**DEPC treated H2O:**

* 1 mL DEPC
* 1 LMilliQ 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.*

**PROBES & HAIRPINS DESIGN AND DILUTION**

1. Design probes using Ozpolat lab python script: [*https://github.com/rwnull/insitu\_probe\_generator/releases*](https://github.com/rwnull/insitu_probe_generator/releases)
   1. Order each probe divided in 5-40 pairs (20-30 pairs ideally)

*(Each pair covers 54 bp)*

* 1. Target mRNA length ideally 700-1000 bp.
  2. Avoid low complexity regions and GC-rich regions

(*Increase background amplification)*

1. Order probes (**50 pmol**/each) and hairpins from IDT.

*(Allow 1 week-1 months for manufacture and arrival)*

1. **Dilute probes** adding 50 uL of DEPC-water for a final concentration of **1 pmol/uL**.
2. **Dilute hairpins** in 5xSSC to a final concentration of 3 pmol/uL
3. Store at -20ºC

**PROTOCOL**

**BEEFORE STARTING**

* Make sure DEPC-water has pH 7 approx.
* Always use RNAse free eppendorfs and Falcon tubes
* Always use clean gloves.
* Always use clean Pasteur pipettes.
* Clean your bench and micropipettes with ethanol or RNAse-Zap

(*at least for the 1st day of HCR*).

* Use DEPC-treated solutions to preserve the RNA.

*(at least for the 1st day of HCR)*

* Avoid mechanical shaking/spin down, as it can damage the samples.

*(Animals can be ´moved´ within the baskets by gently submerging a couple of times when changing buffers).*

**FIXATION**

Use DEPC-treated PBSt

1. **UNDER THE HOOD:** Prepare **4% PFA** in **1xPBSt**

*(From pure 16% PFA ampule or from -20ºC frozen 16% PFA leftover)*

*(Don´t use open PFA from the fridge, it acidifies).*

**//OPENING THE AMPULLE:** Under the hood, grab the ampule firmly between both hands and press the lid with the thumbs. Once opened, the ampule is spill-proof.

1. Take *Pristinas* from the MCR white-labelled glass cultures.
2. **Wash** **3x** by transferring the animals to a new recipient with Spring Water.

Shake gently to clean *Pristinas.*

*(When changing to a new recipient, take the minimum culture water possible)*

*(If after washing steps animals are still dirty, rinse them through a green filter using Spring Water).*

1. Relax worms in **ice cold 'Relaxant Solution'** for **15 min (RT)**

*(On a small, clean petri dish)*

*(This step may also help permeabilize the tissue)*

1. Take the animals in Relaxant Solution to the Rowe building.
2. Gently place the animals in epp tubes.
3. Remove as much Spring Water as possible (100 uL left approx.)
4. **UNDER THE HOOD:** Add 10x volumes of **4% PFA** in **1xPBSt** (ex: for 100 uL water left, add 1 mL PFA).
5. Fix for **30-45 min** **(ON ICE)** in the oscillator shaker (speed 7).
6. Using a 1000 uL pipette, **with the tip cut**, gently move fixed animals to an **85 um basket** into a new epp tube.
7. **Wash 4x** for 5 min in **1xPBSt** to remove the PFA **(ON ICE).**
8. Dehydrate through a **Methanol-1xPBSt** series:

***(Only if samples are not used for HCR immediately after fixation)***

* 25% MeOH, 5 min **ON ICE**
* 50% MeOH, 5 min **ON ICE**
* 75% MeOH, 5 min **ON ICE**
* 100% MeOH, 5 min **ON ICE**
* 100%. MeOH, 5 min **ON ICE**

1. Store in methanol at -20ºC

***(Only if samples are not used for HCR immediately after fixation)***

*(****Does not apply for HCR:*** *For traditional in situ, store samples in methanol minimum 1 night. Methanol extracts endogenous alkaline phosphatases and prevents background staining. Longer storage is preferable)*

**HCR DAY 1**

**Containers and volumes:**

Imagen que contiene taza, vidrio, tabla, teléfono

Descripción generada automáticamente

To use throughout the protocol:

* 2 mL epp tubes
* 85 um homemade baskets

*(Move baskets between epp using tweezers)*

* **1 volume** = **500 uL**

***(Unless otherwise is specified, use 1 volume of every reagent, buffer & solution when working with baskets. Larger volumes may overload the basket).***

**Tissue preparation:**

Use DEPC-treated PBSt

1. Rehydrate through a **Methanol-1xPBSt** series:

***(Only if samples are dehydrated)***

* 75% MeOH, 5 min **ON ICE**
* 50% MeOH, 5 min **ON ICE**
* 25% MeOH, 5 min **ON ICE**

1. **Wash 3x** in **1xPBSt**, 5 mins each **(ON ICE)**
2. Prepare N volumes of fresh solutions for:
   1. **Pronase E:** From 10 mg/mL stock to **0.5 mg/mL** final concentration. (Dilution 1:20 in 1xPBSt). **Keep at RT**.
   2. **Glycine:** Prepare **2 mg/mL** final concentration (Dilute 10 mg powder in 5 mL 1xPBSt). **Keep ON ICE.**
   3. **4% PFA:** UNDER THE HOOD, Dilute 16% pure PFA in 1xPBSt. **Keep ON ICE**.

**Pronease digestions:**

1. Digest worms in **0.5 mg/mL** of **Pronase E** for **15 min** **(RT)**
2. Quick wash in 1xPBSt to remove bulk Pronase E **(RT)**
3. **Wash 2x** in **2 mg/ml Glycine** for **5 min** to stop digestion **(ON ICE)**
4. Turn on water bath and set at 37ºC. Thaw probe hybridization buffer.
5. **Wash 2x** in 1xPBSt for 5 min **(ON ICE)**

**Acetylation:**

**UNDER THE HOOD:**

1. Prepare N volumes of 0.1 M triethanolamine. **Keep at RT**

*(Stock 0.2M. Dilution 1:2 with DEPC-water)*

1. Incubate samples for **5 min** in 0.1 M triethanolamine **(RT)**
2. Incubate samples for **5 min** in new 0.1 M triethanolamine + 3 uL/mL acetic anhydride **(RT)**
3. Add another 3 ul/mL of acetic anhydride and incubate for **5 min (RT)**

*(Per sample: 1.5 uL in 500 uL)*

1. Wash 3x for 5 min in 1xPBSt **(ON ICE).**

**Post-fixation:**

**UNDER THE HOOD:**

* Fix in **4 % PFA** for **30 min** **(ON ICE).** Flick the tubes a bit to disaggregate clumps of *Pristina* before post-fixation.
* **Wash 3x** for 5 min in 1xPBSt **(ON ICE).**

**Probes hybridazation:**

1. **Wash 1x** for 5 min in **2xSSC+0.3%CHAPS** to increase salt concentration **(ON ICE)**

*(If CHAPS is not available, use 2xSSC)*

1. **Wash 1x** for 5 min with **50% 2xSSC+0.3%CHAPS** - **50% probe hybridazation buffer** for 5 min **(RT)**.
2. If samples float in the buffer, allow another 5 min to equilibrate.
3. Remove the solution
4. Pre-hybridize samples in **300 µL** of **probe hybridization buffer** for 1 h **(37ºC)**
5. **Prepare probe solution:**

* Add **4 pmol** (**4 µL** because the probe stock is 1 pmol/µL) of **each probe** and fill **up to** **200 uL** with **probe hybridization buffer**.

*(Maximum 4 probes: B1, B2, B3, B4.* ***VIP: Do not add repeated ´Bs´****)*

*(****Control****: 200 uL probe hybridization buffer, with no probes)*

1. Holding the basket with tweezers, add the **probe solution** (200 uL) on top of the **pre-hybridization buffer** (300 uL) to reach a final hybridization volume of **500 µL**. Mix well.
2. Incubate samples overnight (12–16 h) at **37ºC.**

**HCR DAY 2**

To prepare for the day:

* + - Equilibrate **amplification buffer (RT)**
    - Pre-heat **probe wash buffer** in water bath **(37ºC)**
    - Defrost DAPI.
    - Set heat-block at 95ºC.
    - Defrost hairpins **IN THE DARK**

*(B1-H1, B1-H2, B2-H1, B2-H2, B3-H1, B3-H2, B4-H1, B4-H2)*

**Probes washes:**

* + - 1. **Wash 4x** for **15 min** with **probe wash buffer** to remove excess probes **(37ºC)**
      2. **Wash 2x** for **5 min** with **5xSSCt (RT).**

**Amplification:**

Pre-amplify samples with **300 µL** of **amplification buffer** for **30 min** **(RT).**

For each sample, prepare **30 pmol** (**10 µL** because stock is 3pmol/µL) of each **hairpin H1** and **30 pmol** (**10 µL** because stock is 3pmol/µL) of each **hairpin H2** in separate tubes

*(8 tubes in total, do not combine H´s or B´s)*

*(Add hairpins to* ***control sample*** *too)*

To snap cool the hairpins, heat using a thermocycler at **95ºC** for **90 seconds**.

Cool down hairpins to **IN THE DARK** for **30 min** **(RT)**

Prepare hairpin mixture:

* Add all snap-cooled **H1 hairpins** and snap-cooled **H2 hairpins** together.
* Fill **up to 200 uL** (per sample) of **amplification buffer** **(RT)**.
* Add **DAPI** to a final concentration of **2 ug/mL** (stock is 5µg/µL) to stain nuclei.

Cool down the water bath to **26ºC** with ice.

Add 200 uL of hairpin mixture per sample to reach a final amplification volume of **500 µL**.

Incubate overnight (19-20 h) **IN THE DARK,** in a water bath **(26ºC)**

*(Make sure water bath is well covered with aluminium foil)*

**HCR DAY 3**

1. Equilibrate **SlowFade Glass** 1 h prior to use **(RT)**
2. Wash series to remove excess hairpins with **5xSSCt (RT):**

*(****OPTIONAL:*** *Save hairpins for future use)*

* 2x 5 min
* 2x 30 min
* 1x 5 min

**Mounting samples:**

1. Mount samples between two coverslips, one long (24x50 mm) and one short (22x22 mm).
2. Scrape each corner of the small coverslip through **mounting clay.**

*(To mount Pristina, use a minimal amount of clay)*

1. Invert sample basket over a petri dish, using tweezers, and rinse the basket with **5xSSCt** until all samples drop into the dish.
2. Transfer samples to the long coverslip using a pipette.
3. Remove as much buffer as possible.
4. Add a droplet of **SlowFade Glass.**
5. Use the wooden tool to place samples on the slide as straight as possible.

*(Pristina samples lie on their side, curving into a C shape)*

1. Place the short coverslip and gently press down until sample get completely immobilized.
2. Seal the edges with nail polish

*(Double-check there are no sealing gaps after a few hours)*

1. Label every slide with:
   * Experiment number
   * Worm numbers
   * Probes used
   * Hairpins used
2. Place in a slide folder and **keep at 4ºC** until imaging (2-3 months maximum).