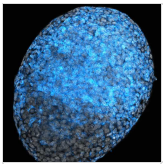

Jun 02, 2021

Hybridization chain reaction (HCR) protocol for Gastruloids (ESC aggregates)

Forked from Hybridization chain reaction (HCR) protocol for tails of mouse embryos

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ABSTRACT

This protocol is for the in situ hybridization chain reaction (HCR) of Gastruloids (3D aggregates of mouse embryonic stem cells). This is an adaptation of the same protocol as applied to mouse tails (Sanchez et al; dx.doi.org/10.17504/protocols.io.7pyhmpw) in turn adapted from the whole-mount mouse embryo protocol available on the website of Molecular Instruments (for details, please follow this link).

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FORK NOTE

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Probe preparation

1 #Note: Steps 1 and 2 only apply if making/ordering your own probes. If buying premade ones from Molecular Instruments, they will come already mixed together and resuspended. Skip to step 3.

Resuspend your probes:

- order each probe at the minimal amount (e.g. 25nmol), Desalted, and Dry
- Upon receipt, spin each tube (e.g. 6000g centrifuge) to collect the pellet to the bottom of the vial
- Add the correct amount of DNAse-free water

To standardise the volumes required in the protocol below, aim to resuspend your probes to X* 20uM, where X=number of primer pairs. As such, if you designed 10 probe pairs, you will resuspend each probe to 10*20=200uM

- Vortex briefly to mix the probes well into solution
- Re-centrifuge to collect all the solution away from the cap
- Store at -20C long term

2 **Prepare ODD and EVEN mixes:**

- in a DNAse-free 0.5mL Eppendorf tube, collect 1uL of each ODD probe
- dilute 1:10 by adding 9 volumes of DNAse-free water

- on a separate DNAse-free 0.5mL Eppendorf tube, collect 1uL of each EVEN probe
- dilute 1:10 by adding 9 volumes of DNAse-free water

By mixing the same volume of each resuspended probe(1uL in this case), the resulting concentration of each probe in the mix will be 1/X of the original one, where X is the number of probes mixed. Because the original probes had been resuspended to X*20uM, the final concentration of each prob in this new mix is 20uM.

By adding 9 volumes of DNAse-free water, the final concentration of each probe in the probe mix is now 2uM.

Worked example:

- I bought 10 probe pairs, and resuspended each to 10*20uM = 200uM, as per Step 1
- I now take 1uL of each of the 10 ODD probes, and collect them in the same tube
- The resulting mix has a volume of 10*1uL = 10uL, and the concentration of each probe is now (1/10)*200uM = 20uM
- I add 9 volumes (9*10uL) of DNAse-free water. The resulting mix has a volume of 100uL, and each probe is now 2uM
- I repeat the same process with the EVEN probes

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- Store at -20C long term
- Independently on the number of primers designed, these ODD and EVEN mixes both have a concentration of 2uM

Day 1: Recovery and fixation of samples

3 Precoat a well of a 6well plate with 2mL serum solution (e.g. PBSFT: PBS+10%FBS+0.2%Triton-X100), for around 30min. Remove the serum solution and replace with with 2mL 4% formaldehyde (**1.08 mL** 37% formaldehyde diluted to **10 mL** with PBS).

To recover samples, use a cut P1000 pipette to pick up each Gastruloid from its well in the 96well plate, collecting all Gastruloids in a 15mL Falcon tube.

Once Gastruloids have sedimented at the bottom of the tube, use a glass pipette connected to a vacuum line to remove as much medium as possible.

Wash quickly by adding 2mL PBS, and transfer all Gastruloids to the pre-prepared PFA-filled well.

Keep in formaldehyde overnight (around **16:00:00**) at **4 °C** in the cold room, with gentle shaking.

Formaldehyde is toxic. Operate under a fume hood and wear appropriate hand, body, and eye protection.

To reduce autofluorescence, it is recommended to use fresh fixative and PBS with no divalent cations (like Ca^{2+} and Mg^{2+}).

Day 2: Dehydration of samples

4 Wash samples with PBST (0.1% Tween20 in 1x PBS).

- Wash 1/2 **00:05:00**
- Wash 2/2 **00:05:00**

5 Dehydrate samples (one sample per tube) with a series of graded methanol : PBST (PBS + 0.1% Tween) washes.

- 25% methanol : 75% PBST **00:05:00**
- 50% methanol : 50% PBST **00:05:00**
- 75% methanol : 25% PBST **00:05:00**
- 100% methanol **00:05:00**
- 100% methanol **00:05:00**

6 Incubate samples at **-20 °C** overnight (around **16:00:00**) or until use.

Day 3: Rehydration of samples and HCR detection stage

7 Rehydrate samples (one sample per PCR tube, capacity = **500 µl** max) with a series of graded methanol : PBST (PBS + 0.1% Tween) washes.

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- 100% methanol **00:05:00**
- 75% methanol : 25% PBST **00:05:00**
- 50% methanol : 50% PBST **00:05:00**
- 25% methanol : 75% PBST **00:05:00**
- 100% PBST **00:05:00**
- 100% PBST **00:05:00**

8 Treat samples with **25 ug/mL** proteinase K (Merck, CAS # 38450-01-6) for **00:04:00** at **Room temperature** .

Proteinase K is sold at a concentration of **18.25 mg/mL** .

Make a **50 ug/mL** solution by diluting **1.37 µl** of Proteinase K in **500 µl** PBST.
Use 1:2 by removing half of the volume from the tube with the samples, and replacing with this new Proteinase K solution.

The resulting concentration of Proteinase K in the tube will be of **25 ug/mL**

9 Wash samples with PBST.

- Wash 1/2 **00:05:00**
- Wash 2/2 **00:05:00**

10 Refix samples in 4% formaldehyde (**2.16 mL** 37% formaldehyde diluted to **20 mL** with PBST) for **00:20:00** at **Room temperature** .

Pre-warm probe hybridization (PH) buffer at **37 °C** for later use (**1.5 mL** /sample).

Formaldehyde is toxic. Operate under a fume hood and wear appropriate hand, body, and eye protection.

Probe hybridization (PH) buffer contains formamide, which is toxic.

To reduce autofluorescence, it is recommended to use fresh fixative and PBS with no divalent cations (like Ca^{2+} and Mg^{2+}).

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11 Wash samples with PBST.

- Wash 1/3 **00:05:00**
- Wash 2/3 **00:05:00**
- Wash 3/3 **00:05:00**

12 Wash samples with PH buffer for **00:05:00** .
To aid later buffer changes, bring Gastruloids to the bottom of the tube by centrifuging **1500 x g, Room temperature , 00:01:00** , or until Gastruloids pellet to the bottom

Probe hybridization (PH) buffer contains formamide, which is toxic.

13 Put fresh PH buffer to the tubes and incubate for **00:30:00** at **37 °C** .

Meanwhile, prepare probe solution by adding 4 pmol (**2 µl** of **2 Micromolar (µM)** stock; since 2uM is 2pmol/uL) of odd probe mixture and 4 pmol of even probe mixture to every **500 µl** of pre-warmed PHP buffer (from Step 8).

We are using a reaction concentration of each HCR probe of 8 nM (8e-9 mol/L, 8e-12mol/mL, = 8pmol/mL)
For a 500 µL reaction volume, each experiment will require 4 pmol of each probe.

probe is sold as 1um = 1pmol/uL , so use 4 uL

Probe hybridization (PH) buffer contains formamide, which is toxic.

14 Reserve part of your samples to a new tube, as negative controls.

Remove PH buffer and add the probe solution. Incubate overnight (around **16:00:00**) at **37 °C** .

Day 4: HCR amplification stage

15 Pre-warm probe wash buffer at **37 °C** (**2 mL** /tube), and equilibriate amplification buffer to **Room temperature** (**1 mL** /tube)

Probe wash (PH) buffer contains formamide, which is toxic.

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16 In separate tubes, for every **500 µl** hairpin mixture, prepare 48 pmol (**16 µl** of **3 Micromolar (µM)** stock; since 3uM stock is 3pmol/uL) of hairpin h1 (tube 1) and 48 pmol of hairpin h2 (tube 2). Snap-cool hairpins by heating the tubes at **95 °C** for **00:01:30** and cool to **Room temperature** in the dark for at least **00:30:00** .

We are using a reaction concentration of each HCR amplifier hairpin of 96 nM. (96e-9 mol/L, 96e-12mol/mL, = 96pmol/mL .
For a 500 µL reaction volume, each experiment will thus require 48 pmol of each hairpin.

probe is sold as 3um = 3pmol/pL , so use 16 uL

17 Wash samples with probe wash buffer at **37 °C** .

- Wash 1/4 **00:15:00**
- Wash 2/4 **00:15:00**

(you can start step 14 during this wash)

- Wash 3/4 **00:15:00**
- Wash 4/4 **00:15:00**

Probe wash (PH) buffer contains formamide, which is toxic.

18 Wash samples with 5x SSCT (0.1% Tween20) at **Room temperature** .

- Wash 1/2 **00:05:00**
- Wash 2/2 **00:05:00**

To prepare **500 mL** of 5x SSCT, dilute **125 mL** of 20x SSC and **0.5 mL** Tween20 to **500 mL** with distilled H_2O.

19 Wash samples with pre-equilibriated amplification buffer (Step 13) for **00:05:00** at **Room temperature** .

Meanwhile, prepare the hairpin mixture (48 pmol of each hairpin in **500 µl** mixture) by mixing the snap-cooled hairpins h1 and h2 (Step 14) to amplification buffer at **Room temperature** .

20 To aid sedimentation of the Gastruloids, centrifuge **1500 x g, Room temperature , 00:00:30**

Remove the amplification buffer from the sample tubes, and add the hairpin mixture.

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Add DAPI [M]2 ug/mL (1:500 dilution of a [M]1 mg/mL stock)

Incubate overnight (around 16:00:00) in the dark at Room temperature .

For dHCR imaging, amplify for a shorter period of time to ensure single-molecule dots are diffraction-limited.

Day 5: Washing, nuclear staining, and imaging

21 Wash samples with 5x SSCT for 00:30:00 in the dark at Room temperature .

22 Stain nuclei with 1:1000 DAPI ([M]2 ug/mL) in the dark at Room temperature .

- Wash + DAPI 1/3 00:25:00
- Wash + DAPI 2/3 00:25:00
- Wash + DAPI 3/3 00:25:00

23 Wash samples with 5x SSCT in the dark at Room temperature

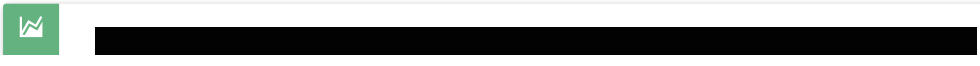
- Wash 1/2 00:15:00
- Wash 2/2 00:15:00

24 Samples are now ready for imaging.

Prior to imaging, the samples could be cleared using the fructose-glycerol clearing solution described in Dekkers et al., 2019. Incubate samples in clearing solution in the dark at 4 °C for at least 02:00:00 .

To prepare the fructose-glycerol clearing solution, dissolve 29.72 g fructose in 33 mL glycerol and 7 mL water on a magnetic stirrer (start the dissolution at least a day before intended day of clearing because it will take a while for fructose to dissolve). The clearing solution can be stored in the dark at 4 °C for at most 1 month. This solution can be used to mount the sample on microscope slide/cover glass. Take note that the clearing is solution has high viscosity.

25 Mount the Gastruloids by using a cut P1000 pipette and transferring them to a 35 µl drop of Fluoromount-G. Cover with a #1 coverslip and wick excess liquid from the sides by using a Kimtech wipe.
Seal all edges with Permount



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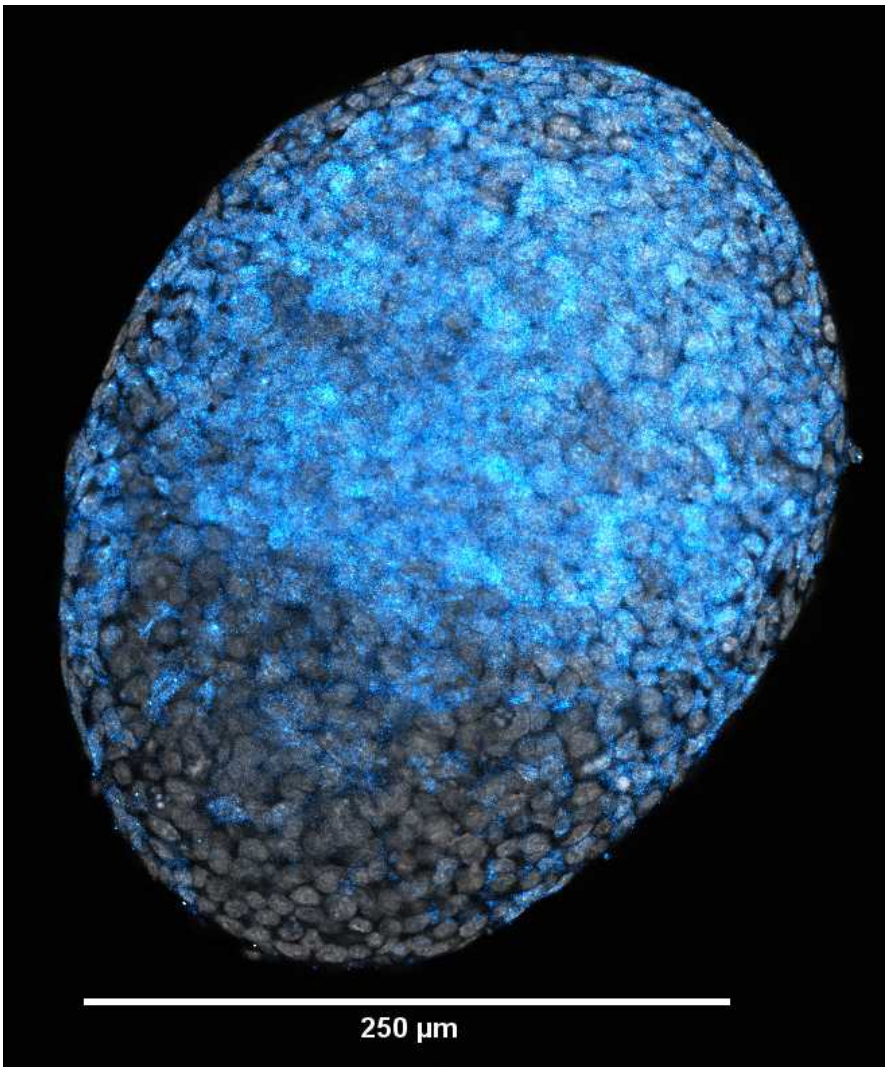

Optical section of a Gastruloid at t=96h, stained for TBra (cyan hot) with HCR. Notice the polarisation of TBra expression to one pole of the Gastruloid.

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