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 We use this protocol and it's working

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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](#)).

References

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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

Note

To standardise the volumes required in the protocol below, aim to resuspend your probes to $X \times 20\mu\text{M}$, where X =number of primer pairs. As such, if you designed 10 probe pairs, you will resuspend each probe to $10 \times 20 = 200\mu\text{M}$

- 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

Note

By mixing the same volume of each resuspended probe (1 μ L 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 $X \times 20 \mu\text{M}$, the final concentration of each probe in this new mix is $20 \mu\text{M}$.

By adding 9 volumes of DNase-free water, the final concentration of each probe in the probe mix is now $2 \mu\text{M}$.

Worked example:

- I bought 10 probe pairs, and resuspended each to $10 \times 20 \mu\text{M} = 200 \mu\text{M}$, as per Step 1
- I now take 1 μ L of each of the 10 ODD probes, and collect them in the same tube
- The resulting mix has a volume of $10 \times 1 \mu\text{L} = 10 \mu\text{L}$, and the concentration of each probe is now $(1/10) \times 200 \mu\text{M} = 20 \mu\text{M}$
- I add 9 volumes ($9 \times 10 \mu\text{L}$) of DNase-free water. The resulting mix has a volume of $100 \mu\text{L}$, and each probe is now $2 \mu\text{M}$
- I repeat the same process with the EVEN probes

- Store at -20°C long term
- Independently on the number of primers designed, these ODD and EVEN mixes both have a concentration of $2 \mu\text{M}$

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.

Safety information

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

Note

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.
 - 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 [M] 25 ug/mL proteinase K (Merck, CAS # 38450-01-6) for ⌚ 00:04:00 at 🌡 Room temperature .

Note

Proteinase K is sold at a concentration of [M] 18.25 mg/mL .

Make a [M] 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 [M] 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).

Safety information

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

Safety information

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

Note

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

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

Safety information

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 [M] 2 micromolar (μM) stock; since 2 μM is 2pmol/ μL) of odd probe mixture and 4 pmol of even probe mixture to every 500 μL of pre-warmed PHP buffer (from Step 8).

Note

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 1 μM = 1pmol/ μL , so use 4 μL

Safety information

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 equilibrate amplification buffer to Room temperature (1 mL /tube)

Safety information

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

- 16 In separate tubes, for every 500 μ L hairpin mixture, prepare 48 pmol (16 μ L of 3 micromolar (μ M) stock; since 3 μ M stock is 3pmol/ μ L) 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 .

Note

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 μ L reaction volume, each experiment will thus require 48 pmol of each hairpin.

probe is sold as 3 μ M = 3pmol/ μ L , so use 16 μ L

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

Safety information

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

Note

To prepare 500 mL of 5x SSCT, dilute 125 mL of 20x SSC and 0.5 mL Tween20 to 500 mL with distilled H₂O.

19 Wash samples with pre-equilibrated 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.

Add DAPI 2 µg/mL (1:500 dilution of a 1 mg/mL stock)

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

Note

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 .

Note

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

Expected result

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.