AQUA-FAANG – Standard Operating Protocol **Chorion removal and cellular isolation of turbot embryos**

ACKNOWLEDGMENTS

This protocol has been developed by Paula Suárez-Bregua and Josep Rotllant at FishBioTech Lab (Department of Biotechnology and Aquaculture. Institute of Marine Research IIM-CSIC), financed by project AGL2017-89648-P (Ministerio de economía, industria y competitividad, Gobierno de España). The protocol has been adapted for the AQUA-FAANG project by Óscar Aramburu González, Belén Gomez Pardo and Paulino Martinez Portela at University of Santiago de Compostela, based on the protocol from Roslin Institute "Roslin SOP ATACseq protocol part 1".

https://data.faang.org/api/fire_api/expeiments/ROSLIN_SOP_ATACseq_protocol_part1_20210527.pdf

OVERVIEW

This protocol describes a method used to extract turbot embryos using a pronase treatment to weaken and eliminate the egg's chorion, which can be difficult to remove manually due to its hardness, plus it can interfere with the follow-up cell isolation and downstream library preparations for ATAC-seq.

IMPORTANT NOTES

Pronase treatment should be carefuly adjusted based on the targeted species, incubation temperature, developmental stage and pronase stock being used for the experiment. We thoroughly recommend a trial treatment using different pronase concentrations (<3mg/ml) and monitoring the status of the eggs at regular intervals, until signs of chorion weakening can be seen. For turbot eggs, when the chorion has been weakened by pronase, they can lose some roundness and start to look deflated in some areas. By this time chorion should be weakened enough in most of the eggs, but the exposure of the embryos to pronase should have been minimal. The duration of the treatment can be extrapolated for future treatments, should any of the conditions of the experiment (species, egg batch, developmental stage, temperature, pronase) do not vary too much.

EQUIPMENT

- 15 ml tubes
- 50 ml tubes
- 1.5 ml tubes
- Petri dishes
- Pasteur pipettes
- Oscillator System for Petri Dishes
- Hula Mixer Tube Rotator System
- Single channel P1000 pipette (suggested)

- Loupe/stereomicroscope (dark background suggested)
- Haemocytometer + cover slips
- Microscope

REAGENTS

- Pronase from *Streptomyces griseus* (ROCHE) REF 10165921001
- 0.25% Trypsin EDTA (for late-stage embryos only; SIGMA) REF T4049-500ML
- Heat inactivated FBS (for late-stage embryos only; GIBCO) REF 10500064
- Sterile MiliQ water
- Cold 1x PBS
- Trypan blue

PREPARATIONS

- Prepare a 30 mg/ml pronase stock solution in sterile MiliQ water. Solution should be kept refrigerated (4-8ºC) with a shelf life of 2-3 days.

- Prepare a 2 mg/ml pronase "ready to use" solution in cold 1X PBS.

  NOTE: The prepared volumes should be adjusted based on the number and capacity of the containers used for the pronase treatment. For this protocol we will be referring to 9 cm diameter petri dishes and their respective volume.

Petri dish size	Petri dish volume	Number of dishes	2 mg/ml "ready to use" pronase volume	30 mg/ml pronase stock solution volume
9 cm diameter	18 ml	3	54 ml	3.6 ml
6 cm diamenter	12 ml	3	36 ml	2.4 ml

1. EMBRYO EXTRACTION

1. Prepare four petri dishes worth of eggs (+200 turbot eggs per dish) with tank saltwater. As embryo development will continue for the duration of the treatment, this should be done 2-4 hours before sampling.

2. Using a Pasteur pipette, remove **most** of the water from 3 of the dishes. It is recommended to place the pipette tip completely perpendicular to the dish surface; this should allow water suction, but the eggs will not pass through.

3. Cover the surface of the **dried** dishes with the "ready to use" pronase solution prepared beforehand (for dishes of 9cm diameter, 18ml should suffice) and put them in the oscillator at moderate speed to **gently** shake the eggs for the duration of the treatment, ideally at egg incubation temperature. The fourth plate (with salt water) should also be put into the oscillator as a control. Make sure there are no dry zones in none of the petri dishes during shaking.

NOTE: As mentioned above in the "Important Note" tab, pronase treatments will vary in length depending on temperature, developmental stage, species and pronase stock. Based on our experience, a pronase treatment on turbot eggs at 15-17ºC with 2 mg/ml

Roche pronase (REF 10165921001) should take between 2.5 h and 3.5 h from blastula to late segmentation.

4. When approaching the estimated end of the treatment (after 2-2.5h), monitor the status of the chorion each ~15 min. Transfer a few eggs with a Pasteur pipette to another dish and check their state under the loupe/stereoscopic microscope (dark background recommended) and pipette up and down to check if the chorion has been weakened.

5. If the chorion looks weakened enough (see "important note" at the start of the protocol), proceed. Discard the pronase solution (same approach as in step 2) with a Pasteur pipette and **fill the plate with PBS**. Put the dish in the oscillator for 1 min and wash the dish by discarding the PBS (step 2 approach) and adding ~18 ml cold PBS.

6. Prepare another dish with ~18 ml cold PBS and transfer a fraction of the embryos. Aggressively pipette the eggs up and down with a Pasteur pipette to remove the chorion, until some eggs start to crack open. If the pronase treatment was successful most of the eggs should be openable. Using needles (or a pipette tip), try to push away the dechorionated embryos from any piece of broken chorion or undechorionated eggs.

7. Pick the required number of embryos with a Pasteur pipette and transfer them to 1.5 ml tubes. Using a P1000 micropipette subtract most of the PBS from the tube, then wash again with 1 ml cold PBS (keep in tube). As you can unintentionally pick up some embryos, we suggest that you empty the subtracted liquid in an empty petri dish over dark background and check if any embryo was picked up.

8. Repeat steps 6 and 7 until enough embryos have been collected (Recommended ~150 for earlier stages, ~50 for later stages).

NOTE: With the onset of epiboly, cells will form distinct layers and it will not be feasible to dissociate embryos efficiently by mechanical means without damaging the cells/nuclei. Depending on whether your stage(s) of interest happens before or after the start of epiboly, two different paths are recommended.

2.1. SAMPLED STAGE BEFORE ONSET OF EPIBOLY

Ignore part 2.2. Sampled stage after onset of epiboly.

9. Pipette up and down 10 times. Use a wide bore pipette tip (or cut the end of a normal pipette tip) and a P1000 pipette.

10. Vortex at a slow speed for 15 s. If solution is not homogeneous and clumps are observed, repeat steps 9-10.

11. Add 10 ul of Trypan blue to an empty 1.5 ml Eppendorf tube. Take a 10 ul sample aliquot for cell counting and mix it with the Trypan blue by pipetting up and down 5 times. Asses cell numbers per ml and percentage of live cells using a haemocytometer.

12. Proceed with designated ATAC protocol (In this case, OmniATAC protocol using Frozen Tissue by CIGENE-NMBU).

https://data.faang.org/api/fire_api/experiments/NMBU_SOP_OmniATAC_protocol_20200429.pdf

2.2. SAMPLED STAGE AFTER ONSET OF EPIBOLY

Ignore part 2.1. Sampled stage before onset of epiboly.

13. Remove supernatant and add 1 ml of cold Trypsin EDTA.

14. Rotate in the Hula Mixer for required time (see Table), 50 rpm at room temperature.

Stage	Treatment duration (min)	Notes
Mid gastrula	5 min	Optional, cells can be isolated by vortex and pipetting
Early somitogenesis	5 min	Monitor digestion and increase if necessary
Mid somitogenesis	10 min	
Late somitogenesis	15 min	
Pharyngula	30-60 min	Treatment duration should be longer the closer you are to hatching stage

15. Add 500 ul of cold heat inactivated FBS to stop the treatment (adjust to Trypsin volume at a 1:2, FBS:Trypsin ratio).

16. Centrifuge for 6 min, 500g, 4ºC. Remove supernatant and resuspend in 1 ml of cold PBS.

11. Add 10 ul of Trypan blue to an empty 1.5 ml Eppendorf tube. Take a 10 ul sample aliquot for cell counting and mix it with the Trypan blue by pipetting up and down 5 times. Asses cell numbers per ml and percentage of live cells using a haemocytometer.

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