**Establishment of primary intestinal epithelial cells and leukocytes from the three-spined stickleback, *Gasterosteus aculeatus***

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**Stock** **solutions:**

**0.1M dithiothreitol (highly unstable at RT):** dissolve 155mg of DTT powder in 10mL dH2O DNase free, Aliquot in 1ml tubes, store at -20C.

**Mucus removal solution:** 18.6ml 1X HBSS + 1ml DTT solution+ 0.4ml FBS.

Divide into two 15 ml tubes.

**Epithelial cells recovery solution:** 29.224g EDTA + 0.4ml FBS + 19.6ml HBSS, adjust pH to 7.3 using hydrochloric acid or sodium hydroxide.

**Enzymatic digestion solution**: 1.4 Wünsch units/ml LiberaseTM + 24 U/ml DNase I + 7ml 1X HBSS.

**Steps:**

**Fish dissection**

Prepare one Petri dish on ice with 10 ml of 1X PBS for collecting the tissue.

Prepare Two Petri dishes containing 10 ml of PBS 1X, 0.1% povidone-iodine and, two Petri dishes with 10 ml 1X PBS and place inside the biosafety cabinet to sterilize the intestine

1. After following approved euthanasia procedures, place the fish’s body on ice.
2. Make a ventral incision from the cloaca to the jaw using sharp surgical scissors.
3. Make two lateral incisions just behind the opercular flaps down to the lateral line of the fish.
4. Using two pins, secure the fish on its dorsal side on a dissecting pad.
5. To detach the intestine, make a cut at pyloric caeca on one side and the cloaca on the other side
6. place intestine into a Petri dish containing 10 ml of cold 1X PBS.
7. Using forceps and mini dissecting scissors, open the intestine by making a longitudinal incision.
8. Bring the Petri dish containing the intestine into the biological safety cabinet and wash the intestine by submerging in two successive 0.1% povidone-iodine washes for 5 minutes each.
9. Wash twice for 5 minutes each in a Petri dish containing 10 ml of cold 1x PBS to remove iodine.

**Mucus removal**:

Transfer opened gut in 10ml of mucus removal solution and incubate for 10 minutes at 17 °C on a gyratory rocker for10 minutes.

Resuspend the gut tissue in a fresh 10 ml of mucus removal solution and incubate again incubate for 10 minutes at 17 °C on a gyratory rocker.

 **Epithelial cells recovery and enzymatic digestion:**

**Enzymatic digestion is affected by the temperatures**

1. Transfer the gut tissue to 10ml of epithelial cells recovery solution and incubate for 10 minutes at 17 °C on a gyratory rocker.
2. In order to recover epithelial cells in the suspension, remove the gut tissue from Epithelial cells recovery solution and keep it on ice for enzymatic digestion step. Centrifuge the cell suspension at 300g for 10 minutes at 17 °C.
3. Remove the supernatant and resuspend the epithelial cell pellet in HBSS with 2% FBS and 1% Pen Strep.
4. Transfer the intestinal tissue from step 2 to 7 ml of enzymatic digestion solution, then incubate for 30 minutes at 17°C on a gyratory shaker.
5. Collect and save the cell suspension at 17 °C.
6. Resuspend the remaining intestinal tissue removed from the enzymatic digestion solution in 7 ml of fresh enzymatic digestion solution for a second enzymatic digestion.
7. Incubate for an additional 30 minutes at 17°C on a gyratory shaker.
8. Recover the cell suspension and pool with cell suspensions obtained from step 5 in a 15 ml conical tube and keep at 17 °C.
9. Filter the obtained cell suspension through a 40µm mesh cell strainer into a new tube to remove cell clumps.
10. Centrifuge the obtained unicellular suspension at 300g for 10 minutes at 17°C.
11. Resuspend the cell pellet in 5 ml L15 with 2% FBS and 1% pen Strep.

**Density gradient:**

1. Use double-density leukocyte isolation medium to recover all leukocytes from the cell suspension.
2. In a 15 mL conical tube, add 10 mL of density medium
3. Carefully layer the 5 mL cell suspension onto of the density medium and mixing the two phases.
4. Centrifuge 20 minutes at 750g at 17 °C.
5. After the density centrifugation, one white layer of cells appears between the L15 medium and Ficoll. Aspirate the top layer of the L15 medium.
6. Next, transfer the mononuclear and polymorphonuclear cell layer to a new conical tube, while making sure to not aspirate the Ficoll gradient with the cells. Wash the cells by centrifuging them at 17°C, 300g, with 10 ml of L15 2% FBS and 1% PenStrep,

**Cell seeding:**

Seed the cells into 96 wells plate at a density of 1x106 cells/ml in L15 media with 10% FBS and 1% PenStrep.

**Materials move to the beginning**

Dithiothreitol: Thermo Scientific™ DTT, catalogue number FERR0861.

HBSS: Gibco™ Cell Dissociation Buffer, enzyme-free, Hanks' Balanced Salt Solution. Catalogue number 13-150-016.

EDTA: Thermo Scientific™ Ethylenediaminetetraacetic acid. Catalogue number AC118432500.

DNase: Thermo Scientific™ Deoxyribonuclease I, bovine pancreas. Catalogue number AAJ62229MB.

Liberase™ DL Research Grade, low Dispase concentration. Catalogue number 50-100-3356.

L15: Gibco™ Leibovitz's L-15 Medium. Catalogue number 11-415-064.

PenStrep: Corning™ Penicillin-Streptomycin Solution. Catalogue number MT30002CI.

FBS: Corning™ Premium Fetal Bovine Serum. Catalogue number MT35015CV.

Double density leukocytes isolation medium: Pluriselect Leuko Spin Medium. Catalogue number SKU 60-00091-10

**Equipment:**

Centrifuge**:** Eppendorf 5804/R

Ph meter: Fisher scientific Accumet AE150

Incubator: Cole-Parmer Mytemp mini CO2

Microscope: Leica DMi1

Stereoscope: Leica S7E

Biosafety cabinet: Sterilgard III Advance