Advancing European Aquaculture by Genome Functional Annotation

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D3.1 Standard Operating Procedure (SOP) for *in vitro* and *in vivo* challenges with PAMPs

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Author(s) ***Name and Organisation***	Geert Wiegertjes, Wageningen University Sam Martin, University of Aberdeen		
Reviewer(s)	Pierre Boudinot		
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Short description	Standard Operating Procedure (laboratory protocol) for in vitro and in vivo challenges with PAMPs to obtain material for epigenetic analysis		

Change Records			
Version	Date	Changes	Responsible
0.1	29.01.2020	First draft	Geert Wiegertjes
1.0	30.01.2020	Minor specifications to protocols (page 4 and page 6). Corrected spelling errors.	Geert Wiegertjes

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Content

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1 Executive summary

This deliverable describes a standard operating procedure (SOP) as a set of step-by-step instructions. The purpose of this document is to assure that all partners involved in WP3 carry out complex routine operations in the same manner, independent of fish species. Thereby this SOP aims to achieve efficiency, quality output and uniformity of performance, while reducing miscommunication. Its main purpose is to achieve the best possible material collection using the most comparable procedure.

2 Primary culture of fish leukocytes from Head Kidney

Required equipment and reagents

For fish dissection

- One polystyrene box with ice
- Balance
- Scalpels, small tweezers, spatula and scissors
- 50ml tubes
- Marker, pen and notepad
- 70% Ethanol in spray flask
- Absorbent paper

For cell culture

- L15 culture medium (e.g. Gibco catalogue number 11580396)
- DMEM culture medium (e.g. Gibco catalogue number 11965084)
- RPMI (e.g. Gibco catalogue number 11875085)
- EDTA pH8, 0.5M (e.g. Invitrogen catalogue number AM9260G)
- Fetal Bovine Serum (FBS) needs to be heat inactivated - sometimes the company will do for you (e.g. Lab tech e.g. catalogue number SKU FCS-SA/500). In principle any heat inactivated FBS is equivalent for this SOP. Preferably, use FBS that has been tested and validated for cells of the fish species considered.
- Antibiotics Pen/Strep (e.g. Gibco catalogue number 11548876)
- Percoll density gradient media (Sigma catalogue number P1644 1L)
- 10 x Hanks Balanced Salt Solution HBSS (e.g. Gibco catalogue number 11570476)
- 7.5% $NaHCo_3$ (e.g. Sigma catalogue number S8761; 100 ml)
- Molecular grade water
- Sterile Syringe (2 ml) – the plunger is used to help the cells through the nylon mesh)
- Sterile 10 mL sterile pipettes
- Sterile 25 mL sterile pipettes
- Sterile 50 mL plastic tubes
- 100 µm Easy strainers for 50 ml tubes (e.g. Greiner bio-one catalogue number 542000)
- Poly I:C ssRNA viral mimic (Sigma catalogue number P1530)
- Vibrio extract bacterial PAMP (from INRA)
- 6-well plates (e.g. Fisher catalogue number 140675)

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Media recipes

For all media, store at 4°C for a maximum period of 2 months

Extraction media

- 500 mL cell culture medium L15, or RPMI, or DMEM (as usual to the individual lab for each fish species)
- 10 mL FBS (2%)
- 0.02% EDTA final conc with EDTA pH8 (e.g. e.g. Sigma catalogue number 93283)

Washing media – prepare in a 50 ml tube

- 50 mL L15
- 50 µL FBS (0.1%)

Growth media – make 500 ml

- 500 mL L15 / RPMI / DMEM
- 10 mL FBS (2%)
- 50 µL Pen/Strep solution

51% Percoll:	**(50 ml)**	**10 ml**
- H_2O	18.9 mL	3.78 mL
- Percoll	25.5 mL	5.0 mL
- 10xHBSS	5 mL	1 mL
- $NaHCO_3$	357 µL	75 µL

3 Preparation of Pathogen Associated Molecular Patterns (PAMPs)

Preparation of Poly I:C

Prepare a **working stock** of poly I:C (Sigma catalogue number P1530) at 5 mg/mL in PBS and store aliquots at −20°C. Before use, aliquots must be heated to 55°C for 15 min and then allowed to cool to room temperature for 20 min. (final working concentration in cells will be 50 µg mL^{-1}).

Preparation of Vibrio extract

One batch of Vibrio extract will be prepared at INRA and distributed to all partners (sent on dry ice). Store at −80°C until use.

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4 Preparation of cells (i.e. leukocytes) from head kidney

Fish Dissection

1. Sacrifice a fish according to approved local regulations.
2. Bleed the fish as completely as possible by cutting gills and blood should emerge from the aortic arteries going into gills, let the fish bleed for about 3 minutes. This procedure is different from the procedure described for WP1 because it is essential to remove as many of the red blood cells (RBC) as possible.
3. To reduce the risk of bacterial contamination, thoroughly spray the fish with 70% Ethanol before carefully opening the abdomen with scissors and knife.
4. Dissect out the entire head kidney (using scalpel, tweezers and spatula) and put into a 50 mL tube (tube 1) containing 20 mL **extraction media.**

Procedure for cell extraction

5. Take a 100 µm nylon mesh and fit it onto a new 50 mL tube (tube 2).
6. Add 45 mL **extraction media** to a new 50 mL tube (tube 3)
7. Using a sterile pipette or tweezers, transfer the head kidney from the tube 1 and place it on nylon mesh.
8. Working gently, squeeze the head kidney though the 100 µm nylon mesh using the rubber end of the plunger from a 2 mL sterile syringe. Continually apply **extraction media** from tube 3 to prevent cells from drying out and assist with their transit through the mesh. NOTE: Be very gentle, head kidney tissue will break up quite easily but this may take 10 minutes.
9. Make the final volume of the disrupted cell suspension to 40 mL using additional **extraction media** from tube 3.

Separate leukocytes from erythrocytes using a percoll gradient

This is a delicate procedure and may require some practice.

10. Pipette 5 mL of 51% percoll into a 50 mL tube.
11. VERY gently layer 20 mL of the cell suspension from Step 9 onto the top of the percoll without causing the layers to mix.
12. Spin the tube for 30 min at *400g* in a centrifuge pre-cooled to 4°C. NOTE : Do not use a break on centrifuge! It will take approximately 10 min to stop.
13. Handling the tube carefully so as not to disturb the stratification, use a 10 mL pipette or similar to carefully remove cells at the interface between Percoll and extraction media (these are the desired leukocytes) and transfer to a new 50 mL tube, expect to remove approx. 5 mL from the cell layer at interface. RBC will have pelleted to bottom of tube.
14. Adjust the volume of the collected leukocytes to 20 mL with **washing media.**
15. Centrifuge for 10 min at *400g* and at 4°C to collect the cells.
16. Decant the wash medium carefully, making sure not to discard the cell pellet. Repeat steps 14 and 16 once more to eliminate percoll completely.

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17. Count the cells using haemocytometer and dilute to $1x10^6 mL^{-1}$ and check viability with trypan blue (should be >90% viable). It is required to have a min $18x10^6$ cells per fish for the 3 stimulations.

5 Stimulation of cells (i.e. leukocytes) *in vitro*

For the stimulation *in vitro* n = 6 biological replications of each stimulation are required for analysis.

1. Add 2 mL of cells ($2x10^6$ cells) per well in 1.5 x 6-well plates (9 wells).
2. Ensure you get a min number of $18x10^6$ cells per fish for the 3 stimulations (control, Poly I:C and Vibrio extract).
3. We need minimum of $6x10^6$ cells for 3 wells of the 6-well plate per fish (see table below).
4. This can be scaled up while maintaining the same cell densities if total cell number is > $18x10^6$.

Assay:	Control 3 wells per fish	Poly I:C 3 wells per fish	Vibrio extract 3 wells per fish
RNA-Seq	2 x 10^6 cells	2 x 10^6 cells	2 x 10^6 cells
Small RNA-Seq	2 x 10^6 cells	2 x 10^6 cells	2 x 10^6 cells
ChIP-Seq & ATAC-Seq*	2 x 10^6 cells	2 x 10^6 cells	2 x 10^6 cells

** ChIP and ATAC samples are combined because ATAC requires approx 100.000 cells.*

Poly I:C stimulation

5. Take an aliquot of **working stock** poly I:C and heat to 55°C for 15 min, cool to room temperature for 20 min.
6. Final working concentration in cells will be 50 µg mL^{-1}, which is 20 µL of stock to 2 mL of cell media). Ensure PAMP is well mixed with cells by pipetting gently.

Vibrio extract stimulation

7. Add 20 µL Vibrio extract (from INRA) to 2 mL of cells in growth media. Ensure the PAMP is well mixed with cells by pipetting gently.

Duration of stimulation

Incubate cells in vitro as long as is considered the optimal time for the species (for example, salmonids = 24h at 20°C)

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6 Collection of DNA or RNA post stimulation

WARNING: Cells (especially myeloid) can attach to the bottom and other cells will still be in suspension.

1. After stimulation, harvest **all** cells by also softly removing attached cells with a scraper and then collect all cells in 2 ml Eppendorf tubes.
2. Pellet cells at 5 min x 500*g*

3. For *RNA-Seq* aspirate all medium and flash freeze dry pellet and move to -80°C
4. For *small RNA-Seq* aspirate all medium and flash freeze dry pellet and move to -80°C
5. For *ChIP-Seq* aspirate all medium and flash freeze dry pellet and move to -80°C

NOTE: For ATAC-Seq ensure cells are dissociated by pipetting and transfer 2x100 µl cells (2x100,000 cells) to 2 new Eppendorf tubes. The remainder of cells (1.800.000 cells) from these wells will be processed for ChIP-Seq.

6. For *ATAC-Seq*
    a. Tube 1, 100,000 cells; aspirate all medium and flash freeze dry pellet and move to -80^{o}C AND
    b. Tube 2, 100,000 cells; follow most recently-optimized procedure ATAC-seq (still in progress and optimization, check with University of Aberdeen)

7. For all:
   Proceed – when convenient – with the relevant downstream WP1 protocols for extraction of RNA and of small-RNA, isolation of chromatin for ChIP-Seq and isolation of DNA for ATAC-seq.

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7 Stimulation of head kidney tissue *in vivo*

For the stimulation *in vivo*, n = 6 biological replications of each stimulation are required for analysis.

Preparing PAMPs for *in vivo* challenge:

- Using the 5 mg/ml Poly I:C prepared as described at Step 5-5. Inject intraperitoneally (i.p.) 5 µg per g of fish. Poly I:C prepared in l00 µl PBS.
- Using the Vibrio extract obtained from INRA, combine 90 µL PBS with 10 µL Vibrio extract. Inject intraperitoneally (i.p.)
- Inject 100 µL PBS i.p. as a control vehicle.

- *Fish should be anaesthetized until relaxed prior to PAMP injections.*
- *Fish should be fasted for ≥12h prior to i.p. injection. Maintain fish at the appropriate temperature for each species, with no feeding during the stimulation.*
- *The head kidney will be sampled at maximal immune response time post injection (e.g. 24h for trout, salmon and carp; may be adjusted per fish species)*
- ***Material for all 4 assays will be prepared from head kidney from each individual fish.***

Storage of materials until processing

Head kidney tissues can be flash frozen on dry ice and then moved to -80°C to proceed as described for head kidney tissue sampling in WP1.

Freeze aliquoted tissue sample, if possible n=2 for each down-stream procedure (i.e. ChIP, ATAC, RNA, smallRNA), per treatment, per fish.

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