WUR common carp protocol modifications

to

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

this protocol is a modification of the core protocol “in vivo in vitro challenges with PAMPS” from the institute INRA established for the project AQUA-FAANG, which can be found at this address:
https://data.faang.org/api/fire_api/experiments/INRA_SOP_invivo.invitro.challenges_20200131.pdf

please find the steps that have been modified to the application in this modified protocol. Modifications and additional notes are indicated with * and marked in green. modified mediums can be found at the end of this document.

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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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Short description	Standard Operating Procedure (laboratory protocol) for in vitro and in vivo challenges with PAMPs to obtain material for epigenetic analysis		

Change Records			
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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.

*This file contains the adjustments made for common carp. Original protocol: https://data.faang.org/api/fire_api/experiments/INRA_SOP_invivo.invitro.challenges_20200131.pdf Changes made for common carp are indicated by * sign and green markings. Common carp specific mediums can be found at the end of this document*

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

catalogue numbers differ for experiments performed at WUR AFI due to slight modifications of protocol for common carp, see below for explanation

- 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
- 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

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- 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)

Media recipes

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

Extraction media
***Extraction media was adjusted for common carp:**
The basis of this cell culture medium is DMEM +L15

Homogenization buffer:
- 50mL incomplete NMGFL-15 medium
- 200uL heparin (final concentration 20U/mL)
- 500uL pen/strep (stock = 100x)

Full medium description is mentioned at the end of this document*

Washing media – prepare in a 50 ml tube
For common carp wash was done with homogenization medium

Growth media – make 500 ml
For common carp medium B was used for growth media, (50% DMEM and 50% L15) supplemented with 2% FBS and pen/strep. Description of medium is mentioned at the end of this document

51% Percoll
in common carp:
-51mL Percoll
-44mL incomplete NMGFL-15 medium
-5.1mL10X PBS

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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.

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

Fish Dissection

1. Sacrifice a fish using approved local regulations.
2. Bleed the fish as completely as possible. 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 10 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 20mL using additional **extraction media** from tube 3.

Separate leukocytes from erythrocytes using a percoll gradient

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This is a delicate procedure and may require some practice.

10. *For common carp: Pipette 3mL to four 15mL tubes.*
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. *For common carp this will result in four 15ml tubes containing 3mL of Percoll and 5mL of cells*
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 15 and 16 once more.
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.
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 > $18x10^6$.
   For common carp this was scaled up. The amount of cells was kept the same per sample, only extra replicates were added. For the RNA 5 well with $2x10^6$ cells were used. As total $1x10^7$ cells were used for RNA extraction

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
ChIP-Seq	2 x 10^6 cells	2 x 10^6 cells	2 x 10^6 cells
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. Therefore, if cells are limiting, the remainder of the cells can be used for ChIP seq, this requires an extra handling to separate these cells but this leaves more remaining cells that can be used for RNAseq and sRNAseq*

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For common carp ATAC and ChIP were separated. For ATAC 2M cells/well were seeded. After incubation cells were counted, 75.000 cells were selected for ATAC assay.

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 5 uL 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, resuspend pellet in 350uL RLT buffer and store at -80°C
   *For common carp the RNeasy kit of Qiagen was used. Protocol can be found here: https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/rna-purification/total-rna/rneasy-kits/ *

4. For *ChIP-Seq* aspirate all medium, fix the cells with 1mL 1% formaldehyde, wash the cells with PBS and store the cell pellets at -80°C
   *For common carp the ChIP-Seq kit from Diagenode was used. Protocol can be found here: https://www.diagenode.com/en/p/chipmentation-kit-for-histones *

NOTE: For ATAC-Seq ensure cells are dissociated by pipetting and transfer 2x100 ul 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.
For common carp this was NOT done due to different wells for ChIP and ATAC

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

*For common carp the start point was 75.000 cells. Accordingly step 6 till 14 of protocol: https://data.faang.org/api/fire_api/experiments/ABDN_SOP_fresh_ATAC_20210518.pdf was followed.
Next the switch was made to protocol: https://data.faang.org/api/fire_api/experiments/NMBU_SOP_OmniATAC_protocol_20200429.pdf.
Starting at step 16 this protocol was followed till the end.*

6. 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:

For common carp all stimuli were injected 100µL/100 gram of fish. The fish were estimated at 400g, thus 400 µL was injected.

- Using the 5 mg/ml Poly I:C prepared as described at Step 5-5. Inject intraperitoneally (i.p.).
- Using the Vibrio extract obtained from INRA, make 10x Vibrio dilution with PBS. Inject intraperitoneally (i.p.)
  For common carp a 10x dilution of vibrio was used instead of 50x dilution.
- Inject 400 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 species appropriate temperature, 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 aliquotted tissue sample, at least n=2 for each down-stream procedure (i.e. ChIP, ATAC, RNA, smallRNA), per treatment, per fish.

For common carp this was adjusted. The vivo experiment samples were treated the same as the vitro experiment. A single cells suspension was made of isolated head-kidney leukocytes (described in vitro experiment segment in this file) The cells were processed as described in paragraph 6.

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Cell culture media used at WUR

Medium A — Total volume: ±896 mL

1.	Milli-Q H_2O	700 ml		(machine)
2.	HEPES (sigma)	7.00 g		(powder)
3.	KH_2PO_4	0.688 g		(powder)
4.	K_2HPO_4	0.570 g		(powder)
5.	NaOH	0.75 g		(powder)
6.	$NaHCO_3$	0.34 g		(powder)
7.	**Hanks balanced salt solution** (10x, no Ca^{2+} and Mg^{2+})	80 ml		
8.	MEM amino acid solution (50x)	25 ml		(solution 4°C)
9.	MEM non-essential amino acid solution (100x)	25 ml		(solution 4°C)
10.	MEM sodium pyruvate (100x)	25 ml		(solution 4°C)
11.	MEM vitamin solution (100x)	20 ml		(solution -20°C)
12.	**Nucleic acid solution** (2.5 mM)	20 ml		(solution -20°C)
13.	L-glutamine		0.584 g	(powder)
14.	Insulin 0.010 g (make 10 mg insulin with 1 mL, so take 1 mL of this)			(solution -20°C, in the general chemicals)

10x Hank's balanced salt solution — **Dissolve the salts -> filter sterilize -> store at 4°C**

Per	500	mL
KCl	2.0 g	
KH_2PO_4	0.3 g	
NaCl	40 g	
$Na_2HPO_4.2H_2O$	0.45 g	
D-Glucose	5.0 g	

Nucleic acid solution: 2.5 mM each of adenosine, cytidine, hypoxanthine, thymidine and uridine.

Per 100 ml

0.067 g Adenosine

0.061 g Cytidine

0.034 g Hypoxanthine

0.061 g Thymidine

0.061 g Uridine

Dissolve by vortexing -> incubate in 37°C for 15 minutes -> vortex -> filter sterilize -> store aliquots of 20 mL at -20°C

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50% DMEM / Leibovitz's media, medium B

1. Take 1 L of Leibovits's L-15 medium
2. Add powder for 1L of DMEM
3. Top-up to 2 L with milli-Q H2O
4. Cover with foil and allow to mix using magnetic stirrer.
5. You need 1 L of medium B to make incomplete medium, so filter sterilize the remaining 1 L and store at 4°C

NMGFL-15 (incomplete) medium

1. Make medium A
2. Add 1L medium B (50% DMEM / Leibovitz's)
3. Allow contents to mix on magnetic stirrer.
4. Fill up to 2L with milli-Q H_2O.
5. Adjust pH to 7.4
6. β-mercaptoethanol 7 µl
7. Filter sterilize
8. Store at 4°C

Complete NMGFL-15

1. Add 1 ml gentamicin / 1l of incomplete media (gentamicin 50 mg/ml)
2. 10% Foetal calf serum
3. 5% pooled carp serum
4. 1% pen/strep (100x)

Homogenization buffer

1. 50 ml incomplete NMGFL-15
2. 200 µl Heparin solution (end conc of 20 U/ml)
3. 500 µl Pen/Strep (100X conc.)

51% Percoll

1. 51 ml Percoll
2. 44 ml incomplete NMGFL-15
3. 5.1 ml 10x PBS

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