DTU Aqua
National Institute of Aquatic Resources

EUROPEAN UNION REFERENCE LABORATORY FOR FISH AND CRUSTACEAN DISEASES

Inter-laboratory Proficiency Test 1, 2019

Kgs. Lyngby, 23th September 2019

Dear colleague(s),

The Annual Inter-laboratory Proficiency Test **2019** consists again this year of two separate tests, designated 1 and 2 respectively. **This letter provides information on proficiency test 1 (PT1), only**.

PT1 consists of 5 ampoules (labelled I – V). Each ampoule contains 0.2 ml lyophilised cell culture supernatant mixed in equal volumes with 2% w/v lactalbumin hydrolysate solution. The ampoules should be handled as described in Annex 1 and should be stored in the dark at 4°C after receipt if the solving of the test is not started right away. Beware that the ampoules may contain high titered virus and as such there is a risk of contamination.

Please acknowledge receipt of the parcel immediately on arrival (by email to tevk@aqua.dtu.dk).

In **PT1**, participants are asked to identify all pathogen listed in Council Directive 2006/88/EC) according to the diagnostic procedures included in Commission Implementing Decision (EU) 2015/1554 or in the OIE Aquatic Manual. This means that participants are asked to **isolate and identify the fish viruses causing the notifiable diseases: viral haemorrhagic septicaemia virus (VHSV)** and **infectious hematopoietic necrosis virus (IHNV)** and **to isolate any Rana-virus if present; furthermore the rana-virus isolate has to be further characterized in order to determine whether it is the listed pathogen epizootic haematopoietic necrosis virus (EHNV) or another representative of the ranavirus family.** Participants should be aware that ampoules can also contain other viruses (e.g. other fish rhabdoviruses as perch rhabdovirus or spring viraemia of carp virus (SVCV), birnaviruses as infectious pancreatic necrosis virus (IPNV) or ranaviruses) furthermore the ampoules can contain more than one virus or no virus at all. Beware that the ampoules may contain high titre virus and therefore there is a risk of contamination.

Participants are encouraged to use their normal laboratory procedures following Commission Decision (EU) 2015/1554 using monolayered cell cultures followed by e.g. neutralisation test, immunofluorescence, ELISA or PCR. If ranaviruses should be present in any of the ampoules, it is mandatory to perform a sequence analysis of the isolate in order to determine if the isolate is EHNV.

Since monolayered cell cultures are important tools to be used for fish health surveillance for VHS, IHN and EHN, and for isolation of the respective viruses, we ask each participant to perform a titration of the content of each ampoule according to the procedures described in Annex 2. This ensures that the same procedures are used in all laboratories, making a comparison of the obtained titres – and the sensitivity of the fish cell cultures used - possible. Please record titration and identification results, and make a note on the date of arrival as well as the initial date of testing.
We encourage all laboratories to identify VHSV and IHNV isolates as far as possible by means of genotyping in addition to the standard methods. For VHSV, we suggest to follow the genotype nomenclature and procedures according to Einer-Jensen et al. (2004), Journal of General Virology 85, 1167–1179 ; for IHNV we suggest to follow genotype nomenclature and procedure provided in the latest IHNV chapter of the OIE manual on Aquatic Animal Diseases (primer references are given in Emmenegger et al. (2000), Diseases of Aquatic Organisms 40 (3), 163-176 and PCR conditions are given in Garver et al. (2003), Diseases of Aquatic Organisms 55;187-203) . The genotyping results provided

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from all participants will be compiled and analysed; each participant will receive a separate score in order to assess its capacity of assign a virus isolate to the correct genotype.

The Proficiency test is meant to be a tool for each laboratory to assess its own capacity of detecting and identifying fish pathogens; therefore the content of the ampoules and the results obtained during the examination has to be kept confidential by each participant until the final report has been made available

The results should be returned to us no later than December 1st, 2019. Please be aware that the European Commission will be forwarded your results.

In order to obtain a more uniform way of answering please submit your results in the Excel spread sheet there is available for download at the EURL web page, http://www.eurl-fish-crustacean.eu/. This year the cells for typing in the analysis results in the spreadsheet will be prefilled with N/A and must be overwritten when filling in your results.
Remember to fill the form according to related instructions available on the website – So kindly remember:

- In the tables for titration mark CPE with an "X" and nothing else
- Mark if you have used a cell-line (especially if no CPE is obtained)
- Only fill in the virus name under 'Concluding Results'
- Only fill in the Genotype under 'Genotype'

We would kindly ask laboratories to submit all sequencing results that have been used for genotyping of isolates by using the spread sheet 'Sequencing results' and remember Rana isolates included shall be sequenced in order to distinguish EHNV from the non-listed Ranaviruses.
Within the spreadsheet is included a questionnaire on the accreditation status of the laboratory. We kindly ask all participants to fill the questionnaire.

You are requested to:
• Pt. 1 Ampoule I-V: Virus identification results and virus titration results
• Sequencing results: Sequencing data
• Accreditation situation: Questionnaire on accreditation status

Please submit the filled out spreadsheet in an email to tevk@aqua.dtu.dk

All data will be compiled and a report produced and returned for your information, with the coding of each participating laboratory kept confidential.

We request that you do not forward any virus-isolate which may be present in the received samples of the proficiency tests to third parts without having contacted the EURL-Fish diseases for permission in advance.

For further information please do not hesitate to contact us by telephone or e-mail.

Yours sincerely,

Teena Vendel Klinge, Niels Jørgen Olesen and Niccolò Vendramin

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***Annex 1: Inter-laboratory Proficiency Test 1,* 2019**

The ampoules were produced at the National Institute for Aquatic Resources, Technical University of Denmark in August 2019

Each ampoule contains 0.2 ml cell culture supernatant mixed in a 1:1 ratio with a 2% w/v lactalbumin hydrolysate solution in water (0.4 ml/ampoule).

Please store the ampoules in the dark at 4°C upon receipt, and proceed with the examination at your earliest convenience.

Please be aware, when opening the ampoules the content will tend to escape due to the vacuum in the closed ampoules! For opening the ampoules we recommend you to use the small saw that is included in the shipment,. Opening of an ampoule is done by making a mark/scratch in one side of the ampoule (only one cut with the saw). Then wrap the ampoule in paper (to minimize the risk of cutting your hands on the broken glass) and open by cracking the top of the ampoule in the direction away from the saw marks. As the saw only makes a scratch on the outside of the glass ampoule, a saw can be reused to scratch more ampoules without cross contaminating samples. Re-dissolve the lyophilized material carefully in 2.00 ml cell culture or dilution medium (e.g. Eagles MEM supplemented with 10% foetal or newborn bovine serum and Tris or Hepes buffer), filter the solution through 0.45 µm membrane filter and transfer the solution to sterile tubes. The dilution is to be used directly for titration. We recommend that the dilution is also used for inoculation onto cell cultures in 24-well plates for identification and characterisation.

Use gloves, disinfect and discard all used material carefully between each ampoule in order to prevent cross contamination.

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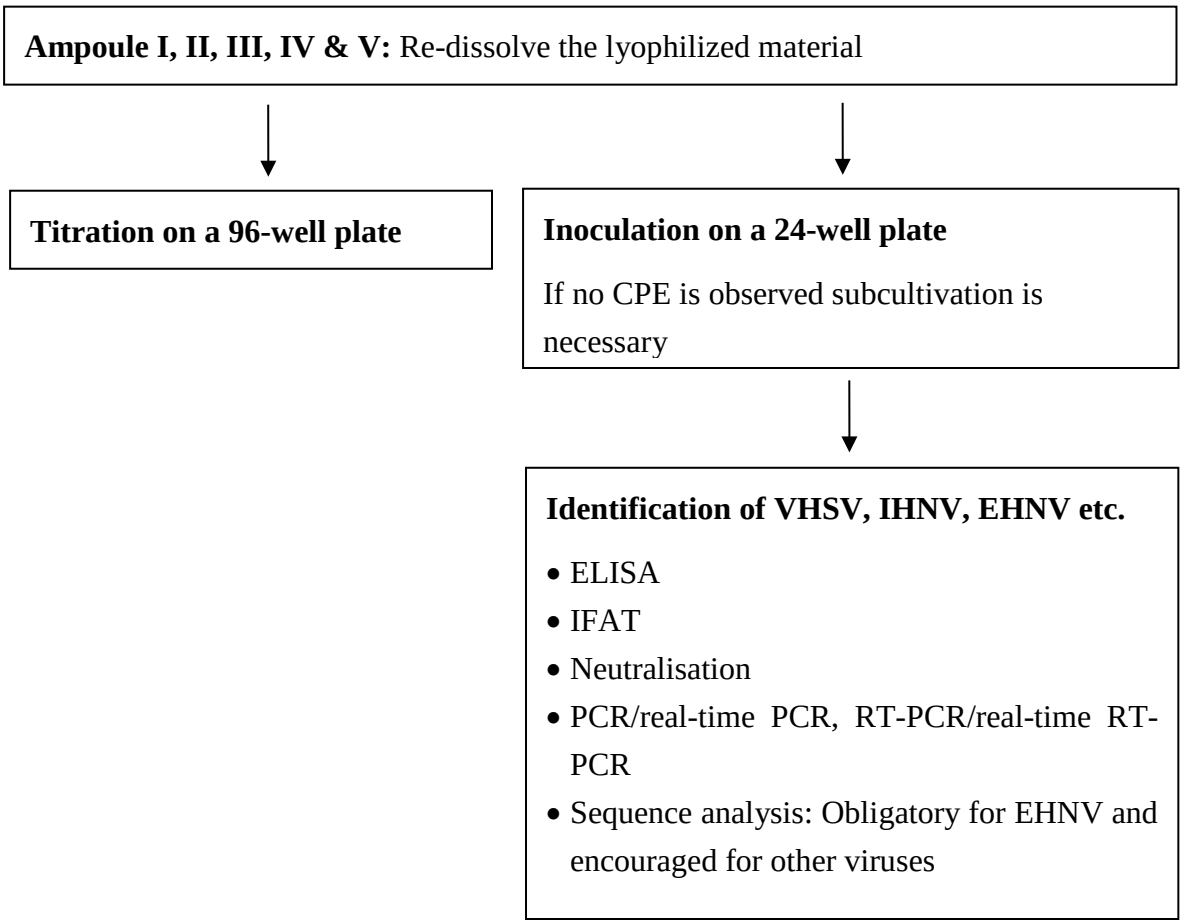
Recommendations for solving the proficiency test PT1



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Annex 2.1 PT1: Titration procedure

Re-dissolve the lyophilized material carefully in 2.00 ml cell culture medium supplemented with 10% foetal (FCS) or newborn calf serum and Tris or Hepes buffer (dilution medium), filter through 0.45 µm membrane filters and transfer the solution to sterile tubes.

For dilution of the content of the ampoules, use one 96-well micro titration plate for each ampoule (e.g. Micro well Plates 262170, Life Technologies).

1. Per cell line to be used for titration: Transfer 180 µl dilution medium into 7 wells in one column (e.g. well B1 to well H1 for BF-2 cells, B2 to H2 for EPC etc., please consult annex 2-2).

2. Transfer 200 µl re-dissolved virus into the appropriate number of wells in row A (e.g., transfer the content of ampoule I into well A1, A2, A3 and A4 if 4 cell lines is used for titration). Ampoule II, ampoule III, ampoule IV and ampoule V is diluted in a similar manner on separate plates.

3. Use a multi-channel pipette for 20 µl volumes for making the dilution. Lower the tip into the undiluted virus in row A, mix 20 times and transfer 20 µl to the surface of the medium in the wells of row B. Put on new tips, lower into wells in row B, and mix 20 times and transfer 20 µl to the surface of the medium in wells in row C. Put on new tips, an continue as described above.

For each ampoule, use a 96-well cell culture plate with 24 hours old BF-2 or RTG-2 cells, and EPC or FHM cells. A seeding density of 50.000 to 100.000 cells per well, in our laboratory, results in approximately 80% confluence after 24 hours incubation at 20°C, but this may vary from laboratory to laboratory. Use normal cell culture medium (e.g. Eagles MEM with 10% FCS, antibiotics, & Tris-buffer), 150 µl per well.

Inoculate 25 µl/well of each virus dilution into 6 replicate wells for each cell line, using a multi-channel pipette. The dilution and inoculation procedure is illustrated on the following page.
Incubate at 15°C until final reading 7 days after inoculation, where wells with CPE are registered.

Please use the Excel spreadsheet to be downloaded from the EURL web page, http://www.eurl-fish-crustacean.eu/ to register the results. Please register the following:

- The titration results (X for those wells with CPE and – for those without, see explanation in the spreadsheet),
- Details of the cell cultures used for the titration – please Mark if you have used a cell-line (especially if no CPE is obtained)
- Identification results and methods in the tables provided in the spreadsheet – please Only fill in the virus name under 'Concluding Results' and only fill in the Genotype under 'Genotype'. All other information shall be included under "Possible isolates".

In order to assure a uniform calculation of titres, all titres will be calculated at the National Institute for Aquatic Resources, Technical University of Denmark (DTU Aqua) based on the raw data presented in spreadsheet to be downloaded from the EURL web page, http://www.eurl-fish-crustacean.eu/

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Annex 2.2 PT1

1. Dilution plate

One plate for each ampoule

			Ampoule I											
Cell line			BF-2	EPC	RTG-2	HFM								
10 fold virus dilution			1	2	3	4	5	6	7	8	9	10	11	12
200µl re-dissolved	10^{0}	A												
20µl re-dissolved	10^{-1}	B												
20µl 10^{-1}	10^{-2}	C												
20µl 10^{-2}	10^{-3}	D												
20µl 10^{-3}	10^{-4}	E												
20µl 10^{-4}	10^{-5}	F												
20µl 10^{-5}	10^{-6}	G												
20µl 10^{-6}	10^{-7}	H												

Transfer 180 µl cell culture medium

96-well plate with 24-hour old cell monolayer		BF-2/RTG-2						EPC/FHM					
		1	2	3	4	5	6	7	8	9	10	11	12
10^{0}	A												
10^{-1}	B												
10^{-2}	C												
10^{-3}	D												
10^{-4}	E												
10^{-5}	F												
10^{-6}	G												
10^{-7}	H					C	C					C	C

C = Control well, cell suspension without virus

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